A HETEROLOGOUS SYSTEM FOR EXPLORING THE LOCALIZATION OF ARABIDOPSIS tRNA NUCLEOTIDYLTRANSFERASE.

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ABSTRACT

A heterologous system for exploring the localization of Arabidopsis tRNA nucleotidyltransferase.

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The tRNA nucleotidyltransferase enzyme is crucial for normal tRNA maturation in eukaryotic cells. Sequence analysis revealed a possible nuclear localization signal (KDTKGKSIPVVNHIFKFSMKRK) in the *Arabidopsis thaliana* tRNA nucleotidyltransferase. We set out to examine the role of this sequence by expressing wild-type and variant Arabidopsis tRNA nucleotidyltransferase proteins in *Saccharomyces cerevisiae*. Viability assays implementing plasmid shuffling and complementation of a temperature-sensitive strain revealed that while the wild-type enzyme could replace the nucleocytosolic role of the yeast tRNA nucleotidyltransferase the variant enzymes could not. Subsequent enzyme activity assays revealed that enzyme activity was significantly reduced for the variant enzymes. Localization studies utilizing tRNA nucleotidyltransferase-green fluorescent protein fusion proteins showed a pattern of distribution inconsistent with the role of the modified region as a nuclear localization signal. In fact, the patterns observed seem to point to a possible stability-modulating function for our candidate region with a marked augmentation in fluorescence in the cytosol and nucleus in the variant proteins. Future experiments will be required to accurately define the role of this sequence in tRNA nucleotidyltransferase structure and function. Furthermore, additional studies are needed to define any nuclear localization signals that may be present in the Arabidopsis tRNA nucleotidyltransferase.

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DEDICATION

I would like to dedicate my thesis to my encouraging father Fouad, beloved mother Dalal, and caring brother Joe, who although had a hard time dealing with my absence, their endless support from miles away instilled motivation in me to persevere.

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ABBREVIATIONS

aa	-amino acid
Ade	-adenine
ATP	-adenosine triphosphate
bp	-base pair
CCA	-cytidine, cytidine, adenosine
CTP	-cytidine triphosphate
Em	-emerald
g	-gram
GFP	-green fluorescent protein
l	-liter
mGFP	-modified green fluorescent protein
mRNA	-messenger ribonucleic acid
OD	-optical density
PCR	-polymerase chain reaction
rpm	-revolutions per minute
SC	-synthetic complete
TBE	-tris/borate/EDTA
TE	-tris/EDTA
tRNA	-transfer ribonucleic acid
Ura	-uracil
YPD	-yeast extract/peptone/dextrose
YPG	-yeast extract/peptone/glycerol

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1. INTRODUCTION

1.1 Transfer ribonucleic acids (tRNAs)

Transfer ribonucleic acids (tRNAs) are essential components of all organisms where they act as adaptor molecules in protein synthesis by taking information contained in messenger RNA (mRNA) and converting it into the amino acid sequence of proteins (Soll and RajBahandari; 1995). Transfer RNAs can be folded into a typical cloverleaf secondary structure with four distinct regions: the acceptor stem, the anticodon stem and loop, the D arm, and the T arm (Fig.1) and have a conserved three-dimensional structure (Soll and RajBahandary, 1995). In addition, all functional tRNAs end in a 3'-terminal cytidine, cytidine, adenosine (CCA) sequence required for aminoacylation (Maizels *et al.*, 1999).

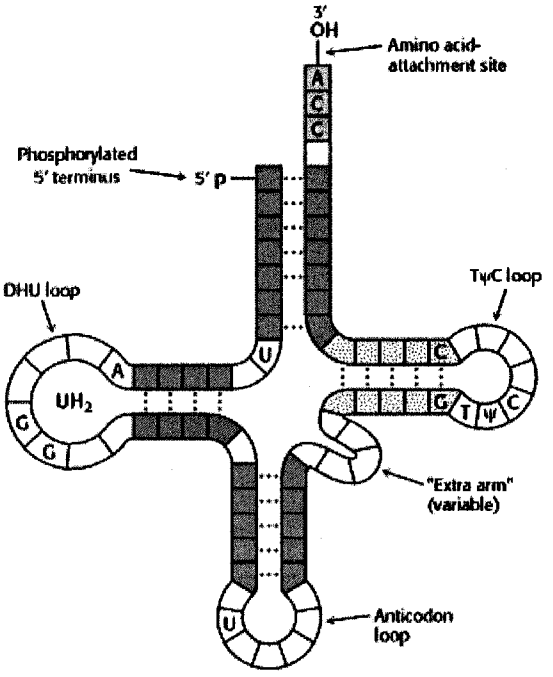


Figure 1: Typical cloverleaf secondary structure of tRNAs (Berg *et al.*, 2002).

1.2 Transfer RNA maturation

The synthesis of mature and functional tRNAs requires many different reactions mediated by multiple enzymes. Initially, tRNAs are produced as primary transcripts containing 5' and 3' extensions and in some cases introns that are removed by specific processing enzymes (Hopper and Phizicky, 2003). In addition to this RNA processing, specific nucleotides within the tRNA may be modified, e.g. methylation, isopentylation, etc. Some base modifications may influence the rate and fidelity of protein synthesis as well as the stability of the tRNA itself (Hopper and Phizicky, 2003). If after 3' end processing a terminal cytidine-cytidine-adenosine (CCA) is not present, it must be added by the enzyme tRNA nucleotidyltransferase (Hopper and Phizicky, 2003).

1.3 Transfer RNA nucleotidyltransferase

Since the 3' CCA sequence is universally conserved and required for protein synthesis, tRNA nucleotidyltransferase is also essential for protein synthesis, and ultimately cell survival in organisms, such as all eukaryotes, whose tRNA genes do not encode this sequence. In contrast, in some prokaryotes, such as *E. coli*, all tRNA genes encode the 3' CCA sequence and therefore tRNA nucleotidyltransferase is not essential although it does have a repair function (Zhu and Deutscher, 1987). The specific mechanism by which the CCA sequence is added to maturing tRNAs has attracted much attention due to the unorthodox manner in which tRNA nucleotidyltransferase is able to

attach this sequence with a high degree of fidelity in the complete absence of a nucleic acid template (Weiner, 2004; Xiong and Steitz, 2006).

A vast amount of work in the fields of enzymology and crystallography has been invested in order to elucidate the exact mechanism by which tRNA nucleotidyltransferase is able to achieve this seemingly magical feat. Studies of primary amino acid sequences and enzyme structure have shown a distinct evolutionary divergence in the development of tRNA nucleotidyltransferases (Okabe *et al.*, 2003). This leads to a subdivision of CCA-adding enzymes into Class I (of archael origin) and Class II (of eukaryotic and eubacterial origin) based on functional motifs and tertiary structural variances (Yue *et al.*, 1996; Xiong *et al.*, 2003; Augustin *et al.*, 2003). The current belief regarding the differences between these two classes is related to the characterization of the nucleotide binding site. In the class I structure, it appears that the presence of the tRNA substrate is integral to the definition of the binding site while in the class II structure a pre-existing nucleotide binding site is able to recognize incoming ATP and CTP molecules which will be bonded to the 3' end of the tRNA (Xiong *et al.*, 2003; Li *et al.*, 2002). Mechanistic studies have shown that tRNA nucleotidyltransferase works by maintaining the tRNA acceptor stem bound to itself while the cytidines and adenosine are added to the RNA. Simultaneously, the elongating 3' end of the tRNA folds back on itself to allow the new hydroxyl group to be accessed by the nucleotidyltransferase motif and incoming nucleotide (Xiong *et al.*, 2003; Li *et al.*, 2002).

Although both Class I and Class II enzymes share sequence similarity in their active site regions with certain specific conserved amino acids (*e.g.*, the DXD and RRD motifs involved in catalysis and nucleotide binding), Class I enzymes show little overall

similarity to one another or to Class II enzymes. In contrast, Class II enzymes show a higher level of sequence conservation confined primarily to their amino-terminal sequences (Yue *et al.*, 1998). These amino-terminal sequences correspond to the so-called head and neck regions defined in the available crystal structures. These head and neck domains accommodate the 3' end of the tRNA and serve as the site of catalysis, while the more carboxy-terminal body and tail domains are mainly involved in binding the tRNA (Okabe *et al.*, 2003; Tomita *et al.*, 2006; Xiong *et al.*, 2003). Recent experiments with the *Bacillus stearothermophilus* tRNA nucleotidyltransferase, where site-directed mutagenesis was used to change the CCA-adding activity to a UUG-adding activity, have highlighted the importance of the head and neck domains for enzyme activity (Cho *et al.*, 2007).

1.4 Localization of tRNA nucleotidyltransferase

In prokaryotes, a lack of internal organelles allows transcription and translation to be coupled and take place simultaneously. In contrast, in eukaryotes these processes are separated with transcription restricted to the nucleus and translation occurring outside the nucleus. While the majority of eukaryotic protein synthesis takes place in the cytosol on free ribosomes or on ribosomes associated with the rough endoplasmic reticulum it also occurs in mitochondria and chloroplasts. So, tRNA nucleotidyltransferase enzymes are required in the cytosol, mitochondria and plastids to generate functional tRNAs. Moreover, aminoacyl-tRNA synthetases have been shown to be functional in the nucleus (Azad *et al.*, 2001) and for this to be the case tRNAs in this location must have a complete 3'-terminal CCA sequence. Despite the lack of protein synthesis in the nucleus,

studies have shown that in *Xenopus* the presence of up to 30% of total tRNA nucleotidyltransferase activity is found in this organelle (Solari *et al.*, 1982). So, tRNA nucleotidyltransferase is found not only in the cytosol, mitochondria and plastids but also in the nucleus.

1.5 Role of tRNA nucleotidyltransferase in tRNA export

For cytosolic protein synthesis to take place, tRNAs must be exported from the nucleus where they are transcribed. This nuclear export occurs through the nuclear pore complex. The nuclear export of tRNAs is energy dependent and requires proteins such as Ran and NXT1 (Ossareh-Nazari *et al.*, 2000; Sarkar and Hopper, 1998). One protein involved in tRNA export from the nucleus in *Saccharomyces cerevisiae* is Los1p (the yeast homologue of mammalian exportin-t) which is similar to the importin α and β families of proteins (Sarkar *et al.* 1999, Feng and Hopper, 2002). When human exportin-t was overexpressed in *Xenopus* oocytes and in HeLa cells it facilitated the export of tRNA from the nucleus to the cytosol (Arts *et al.*, 1998, Kutay *et al.*, 1998). Of particular interest to my project is the fact that a Los1p defect can be suppressed by overexpression of the enzyme tRNA nucleotidyltransferase meaning that an additional mechanism for tRNA export from the nucleus probably exists and includes tRNA nucleotidyltransferase as one of the proteins involved in this export (Feng and Hopper 2002). In yeast, the *LOS1* gene is not essential so there must exist an additional mechanism for tRNA export from the nucleus (Hurt *et al.*, 1987). If this is the case then another export receptor may be present (Steiner-Masonyi and Mangroo, 2004). A large number of proteins are required in

the synthesis of tRNA and its export. It is also possible that some of these proteins will interact with each other and with the tRNA itself (Kruse *et al.*, 2000). The fact that overexpression of tRNA nucleotidyltransferase can suppress a defect in the export of tRNAs from the nucleus suggests that tRNA nucleotidyltransferase may play some role in tRNA export and perhaps that this enzyme is required in the nucleus to facilitate tRNA export in yeast.

1.6 Sorting isozymes

An *International Union of Pure and Applied Chemistry* (IUPAC) committee in 1971 made the recommendation that "multiple enzyme forms" should be known as isoenzymes or isozymes. However, they recommended that the term should only apply to multiple forms of enzymes that differ in their primary amino acid sequence due to genetic manipulation as opposed to any structural differences brought about by changes to the primary genetic sequence of the gene encoding the enzyme in question (IUPAC advisory committee on Isozymes, 1971). More recently (Stanford *et al.*, 2000), the term "sorting isozyme" was coined to describe enzymes which are encoded by single genes yet distributed to multiple subcellular localities based on their differing primary sequence which leads to various targeting signals.

Many tRNA nucleotidyltransferases have provided good candidates for sorting isozymes. For example, in *S. cerevisiae*, one gene (*CCA1*) codes for the nuclear, mitochondrial, and cytosolic forms of tRNA nucleotidyltransferase (Chen *et al.*, 1992). A similar situation exists in the yeasts *Candida glabrata* (Hanic-Joyce and Joyce, 2002)

and *Kluyvermoyces lactis* (Deng *et al.*, 2000) and likely in animals (Nagaike *et al.*, 2001, Keady *et al.*, 2002). In addition, in the plants *Arabidopsis thaliana* (the Arabidopsis genome initiative, 2000), and *Oryza sativa* (Yu *et al.*, 2002, Goff *et al.* 2002) for which whole genome sequences are available, sequence analysis suggests a single gene coding for tRNA nucleotidyltransferase. Therefore, in these organisms tRNA nucleotidyltransferase is likely also a sorting isozyme that is shared between the cytosol, nucleus, plastid, and mitochondrion.

The various isoforms of tRNA nucleotidyltransferase localize to different cellular compartments based on the targeting sequences they possess. Wolfe *et al.* (1994) showed that the targeting to the mitochondrion, nucleus, and cytosol in yeast is controlled by the presence of various transcription start sites upstream of multiple in-frame start codons. It was shown that in the mRNAs resulting from transcription of this gene, any of the three initial ATGs within the open-reading-frame (ORF) could be utilized as sites for beginning translation. By altering where transcription initiated, the AUG used to initiate translation could be changed. Three possible proteins could result, beginning at start sites one, two, or three based on the start codon used. In *S. cerevisiae* Cca1p-I (generated from the longest transcripts initiating upstream of ATG1) is the isozyme which localizes primarily to mitochondria while the shorter Cca1p-II and Cca1p-III proteins (generated from shorter transcripts) appear to be present in the cytosol and nucleus (Wolfe *et al.*, 1994; Chen *et al.*, 1992). In plants such as *Arabidopsis thaliana* multiple transcription and translation start sites are also used with the longer forms of the proteins directed towards the mitochondria and plastids while the shorter form of the protein remains in the

cytosol and could potentially be targeted to the nucleus (Schmidt von Braun *et al.*, accepted).

1.7 Targeting information contained on tRNA nucleotidyltransferase

To begin to define targeting signals contained on eukaryotic tRNA nucleotidyltransferases, sequence comparisons of eukaryotic and prokaryotic tRNA nucleotidyltransferases were carried out (Stanford *et al.*, 2000). From these analyses it became clear that the structures of tRNA nucleotidyltransferases in prokaryotic and eukaryotic cells do differ. In the latter where there is an obvious need for specific signals to direct the isozymes to different compartments, additional protein domains named ADEPTs (Additional Domains for Eukaryotic Protein Targeting) are present. In fact, a survey of predicted protein sequences of nucleic acid-interacting sorting isozymes from eukaryotes and their homologues in archaea and eubacteria showed this to be a common feature of all of these proteins (Stanford *et al.*, 2000). Based on this type of analysis, possible regions to examine for potential targeting information can be defined. Here the alignment of the *Arabidopsis thaliana*, *Saccharomyces cerevisiae* and *Escherichia coli* tRNA nucleotidtyltransferases is presented as an example (Fig. 2).

```
AT   mrlsslpintlinlpkslflispfrfrnlnrsltvasrisstllrvsgvssrpcgywfst
SC   mlrsti-------------------------sllmnsaaqktmtnsnfvlnap-------
EC   ------------------------------------------------------------

AT   naamtnvgeedkqsipsielkenieltdkerkifdrl-----lstlrycnl--dtqlrva
SC   ----------------------kitltkveqnicnllndytdlynqkyhnkpepltlrit
EC   ------------------------------------------------------mkiylv
                                                             : :.
```

```
AT   ggwvrdkllgkesddidiaidnmsgsefld-kfkeylssrdee----vqgdtviernpdq
SC   ggwvrdkllgqgshdldiainvmsgeqfat-glneylqqhyakygakphnihkidknpek
EC   ggavrdallglpvkdrdwvvvgstpqemldagyqqvgrdfpvflhpqtheeyalarterk
     ** *** ***   .* * .:   : .::     ::   .         :    : :.  :

AT   skhletaklriydqwidfvnlrseeytensriptmkfgtakddafrrdltinslfynins
SC   skhletattklfgvevdfvnlrsekytelsripkvcfgtpeedalrrdatlnalfynihk
EC   sgsgytgftcyaapdv----------------------tleddlkrrdltinalaqd-dn
     *    *.                              :* ::*  *** *:*:*  : ..

AT   gavedltergiddlksgkivtplpakatflddplrvlravrfgarf---gftldeelkea
SC   gevedftkrglqdlkdgvlrtplpakqtflddplrvlrlirfasrf---nftidpevmae
EC   geiid-pynglgdlqnrllrhvspa---fgedplrvlrvarfaaryahlgfriadetla-
     * : * . .*: **:.  :    **   * :******* **.:*:   .* :  *

AT   asseevrvalgekisrerigneidlmisgngpvsavtylsdlklfsvvfal--pssaeps
SC   mgdpqinvafnskisrervgvemekilvgptpllalqliqrahlenviffwhndssvvkf
EC   lmremthagelehltpervwketesalttrnpqvffqvlrdcgalrvlf-----------
           ...  .::: **:  * :  :    *   .  :       *:*

AT   ppencgslsqsyleamwsllktprpgkfsge-qrrlalyaamflpfrktvykdtkgksip
SC   neencqdmdkinhvyndniln-------shl-ksfielyp-mfle-klpilrekigrspg
EC   ------------pe-idalfgvpapakwhpeidtgihtl--mtl---------------
                      :: .  :     : :  :     * *

AT   vvnhifkfsmkrktsdaetvmnihqtterfrslipslevkkdveldeltwaadilehwks
SC   fqqnfilsailspmanlqiignpkkkinnlvsvtesi-vkeglklsknd-aaviaktvds
EC   --------smaamlspqvdv-rfatlchdl---gkgltppelwprhhgh-gpagvklveq
            ::         : .  .  ..:     .:   :     .   ..   :  ..


AT   itlndpvipa---tskir-vltgfllrdikdfwrvslltslllsatvdgsndhqdigqld
SC   icsyeeilakfadrsqlkkseigiflrnfngewetahfasl--------sdaflkipkle
EC   lcqrlrvpneird----------larlv---------aef--------hdlihtfpmln
     :     :                 : * .         :.:         :    :  *:

AT   f-qlermretyltveatihelgldkiwdakplvngreimqiaelkggsrlirewq--qkl
SC   tkkiellfqnynefysyifdnnlnnchelkpivdgkqmakllqmkpgpwlgk-in--nea
EC   pktivklfdsidawrkpqrveqlaltsead--vrgrtgfesadypqgrwlreawevaqsv
                :  : :. *    : .  * *:   :  :   *  * .  :  :.

AT   ltwqlaypngtaeeckewm--rdika----krqri---e
SC   irwqfdnptgtdqelithl--kailp----ky------l
EC   ptkavveagfkgveireeltrrriaavaswkeqrcpkpe
         .  .  .  *    :  : * .    *
```

Figure 2: Multiple sequence alignment of *Arabidopsis thaliana* (AT), *Saccharomyces cerevisiae* (SC) and *Escherichia coli* (EC) tRNA nucleotidyltransferases. The sequences were aligned using TCOFFEE::Regular (Notredame *et al.*, 2000) and the results expressed as a clustalw format and optimized by eye. * indicates amino acid identity, : indicates strongly conserved amino acids, and . indicates weakly conserved amino acids. Four blocks of more than twenty-two amino acids that may represent insertions into the eukaryotic sequences (or deletions from the *E. coli* sequence) are indicated in yellow. A potential nuclear localization signal in the Arabidopsis sequence is underlined.

Given that the *S. cerevisiae* protein contains 546 amino acids and the *Arabidopsis thaliana* protein contains 605 amino acids while the *E. coli* protein contains only 412 amino acids it is not surprising that there would be gaps in the sequence alignment. Moreover, as we know that mitochondrial (and plastid) targeting information tends to be amino terminal it is also not surprising that both eukaryotic proteins have amino terminal extensions not seen on the *E. coli* enzyme. In fact, as mentioned before, amino terminal targeting signals for mitochondrial (Chen *et al.*, 1992, Hanic-Joyce and Joyce, 2002, Deng *et al.*, 2000, Keady *et al.*, 2002, Nagaike *et al.*, 2001) and plastid (Schmidt von Braun *et al.*, submitted) localization seem common for tRNA nucleotidyltransferases in many organisms.

What is more interesting in the context of this project are the three internal blocks of more than twenty-two amino acids that are found in the eukaryotic sequences but which are missing from the *E. coli* sequence. The lack of these amino acids in the *E. coli* enzyme indicates that they are not required for enzyme activity and suggests that they define ADEPTs which may be involved in nuclear localization. Given this possibility, these largest ADEPTs were scanned for amino acid motifs characteristic of classical nuclear localization signals (Lange *et al.*, 2007). When this was done a single sequence characteristic of a bipartite nuclear localization signal (Robbins *et al.*, 1991) was found. This sequence (**K**DT**KG**K**S**IPVVNHIF**K**FSM**KRK**) is defined as two stretches of basic amino acids (in bold) separated by a spacer of about ten amino acids (underlined in Figure 2).

1.8 This work

The purpose of this project was to gain insight into the role of this potential nuclear localization signal in the *Arabidopsis* tRNA nucleotidyltransferase. To do this, tRNA nucleotidyltransferase proteins containing these amino acids or variant proteins containing an altered amino acid sequence with the positively charged amino acids replaced in this region of the protein generating the modified sequence, **EDTEGESIPVVNHIFEFSMEME,** were expressed in *S. cerevisiae*. These experiments were carried out in *S. cerevisiae* for a number of reasons. One rationale for using *S. cerevisiae* was that it represented an easily manipulated model system. Constructing suitable plasmids, transforming and culturing *S. cerevisiae* involve the use of well established and simple techniques. Moreover, expressing the plant DNA within the yeast system may allow us to examine two aspects of altering these amino acids at once, namely, the localization of the isozyme and the effect of these changes in the amino acid sequence on tRNA nucleotidyltransferase activity. Importantly, in *S. cerevisiae* the affects on enzyme activity of changing specific amino acids can be measured *in vivo* using viability assays involving plasmid shuffling or available temperature-sensitive strains. This would determine whether the plant enzyme is capable of adequately replacing the biological function of the native *S. cerevisiae* tRNA nucleotidyltransferase. In addition, to address how changing these amino acids could alter enzyme activity, extracts from the temperature-sensitive strain (which lacks significant levels of endogenous functional protein) carrying plasmids expressing Arabidopsis tRNA nucleotidyltransferase could be assayed for tRNA nucleotidyltransferase activity.

Because *in situ* levels of tRNA nucleotidyltransferase are low and no antibodies are available to the plant enzyme, localization studies are carried out using tRNA nucleotidyltransferase-green fluorescent protein (GFP) fusion proteins with fluorescence microscopy. These studies allow us to visualize not only the relative locations of the different *Arabidopsis* isozymes, but also the specific changes in targeting caused by changes to the nuclear-localization-signal-like region. Furthermore, these studies augment parallel studies being carried out in plants for comparative purposes. In addition, they may provide guiding insights as to where researchers should focus their energies in plant cells where the manipulations are more complicated.

Finally, in the absence of any obvious nuclear localization signal in the *S. cerevisiae* protein it was hoped that developing an understanding of the localization of the Arabidopsis protein would help to direct us to any potential nuclear localization signals in the yeast enzyme. Moreover, without any known nuclear localization signal in yeast, we hoped to use the Arabidopsis protein to address the question of whether tRNA nucleotidyltransferase is required in the nucleus in *S. cerevisiae*.

2. MATERIALS AND METHODS

2.1 Strains and growth media

Saccharomyces cerevisiae strains SCDT-6, YPH500 and E189F, and *Escherichia coli* strain XL2-Blue (Stratagene) were used in this work. The relevant genotypes are listed in Table 1. Yeast strain SCDT-6 was provided by Pamela J. Hanic-Joyce. David Kushner supplied the yeast strains YPH500 and E189F. Growth media used are listed in Table 2.

Table 1: Strains

Strain	Organism	Genotype	References
SCDT-6	*S. cerevisiae*	*MATα ade2-1 his3-11,15 leu2-3,112 ura3 trp1-1 can1-100 cca1::TRP1* [pA163-2]	Pamela Hanic-Joyce
YPH500	*S. cerevisiae*	*MATa ura3-52 lys2-801*^{amber} *ade2-201*^{ochre} *trp1-Δ63 his3-Δ200 leu2-Δ1*	Hao *et al.*, 2004
E189F	*S. cerevisiae*	Same as YPH500 but with point mutations resulting in conversion of Glu189 to Phe in the *CCA1* gene product	Shan *et al.*, 2007
XL2-Blue	*E. coli*	*sup E44 hsd R17 recA1 gyrA46 thi relA1 lac*$^{-}$ *F' [pro AB*$^{+}$ *lacI*$_q$ *lacZ Δ M15::Tn10 (tet*^{R}*)]*	Stratagene

Table 2: Growth Media and Solutions

Organism	Medium	Ingredients	Reference
E. coli	YT	0.8% Bacto-tryptone 0.5% Bacto-yeast extract 0.5% NaCl (plates: 1.5% agar) when required, 50 mg/L ampicillicin or kanamycin was added	Sambrook *et al.*, 1989
E. coli	LB	1% Bacto-tryptone 0.5% Bacto-yeast extract 1% NaCl (plates: 1.5% agar) when required, 50 mg/L ampicillin was added	
S. cerevisiae	YPD	1% Bacto-yeast extract 2% Bacto-peptone 2% dextrose (plates: 2% agar)	Sherman, 1991
S. cerevisiae	YPG	1% Bacto-yeast extract 2% Bacto-peptone 3% glycerol (plates: 2% agar)	
S. cerevisiae	Synthetic complete	0.67% yeast nitrogen base without amino acids 2% dextrose 20 mg/L of adenine, uracil, L-histidine-HCl, L-arginine HCl, and L-methionine, 30 mg/ml L-leucine, L-isoleucine, and L-lysine HCl 50 mg/mL phenylalaline (plates : 2% agar) when cooled, added 10 ml/L 100x T-mix	Bai and Elledge, 1996
S. cerevisiae	100x T-mix	20 mg/L L tryptophan 30 mg/L L-tyrosine 200 mg/L L-threonine filter sterilized	Sherman, 1991

Table 2 (continued)

S. cerevisiae	FOA	Solution A: 1.68 g yeast nitrogen base without amino acids, 5 g glucose, 5 mg adenine, histidine, tryptophan, 12.5 mg uracil, 0.25 g FOA (125 mL) Solution B: 5 g of agar (125 mL) Sol. B was autoclaved Sol. A was filter sterilized Added Sol. A to Sol. B	Boeke *et al.*, 1984
	TE	10 mM Tris-HCl, pH 8.0, 1mM EDTA	
S. cerevisiae	PLAG solution	40% PEG4000, 0.1 M lithium acetate, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 15% glycerol	Akada, 2000
E. coli	2X cracking buffer	0.2 N NaOH, 0.5% SDS, 20% sucrose	Barnes, 1977; Sambrook *et al.*, 1989
E. coli	Easy prep buffer	10 mM Tris-HCl, pH8.0, 1 mM EDTA, 15% sucrose, 2 mg/mL lysozyme, 0.2 mg/mL RNase, 0.1 mg/L bovine serum albumin	Auer and Berghammer, 1993
	TBE 5x (4.0 L)	120 g Tris, 62 g Boric acid, 40 mL 0.5 M EDTA pH 8.0	Sambrook *et al.*, 1989
S. cerevisiae	DAPI mounting solution	1 µg/ml DAPI, 1 mg/ml *p*-phenylenediamine, 50% glycerol	Moreno *et al.*, 1991
S. cerevisiae	1 liter PBS (containing 1% Triton X-100)	8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 pH 7.4, (and 100 ml 10% Triton X-100)	Moreno *et al.*, 1991
	Enzyme assay buffer	200 mM Tris-HCl (pH 8.5), 100 mM NH_4Cl, 1mM EDTA, 1mM phenylmethylsulfonyl fluoride (PMSF), 1 mM 2-mercaptoethanol, 10 mM $MgCl_2$	Najarian *et al.*, 1986

Table 2 (continued)

Assay mix	100 mM glycine buffer (pH 9.0), 10 mM $MgCl_2$, 0.4 mM CTP, 1 mM ATP, 0.2-0.6 µl [α^{32}P] CTP (3000 Ci/mmol, 10 µCi/ µl) with or without 20 µM tRNA	Cudny *et al.*, 1978

2.2 Plasmids

All plasmids used in this work are listed in Table 3. Plasmids A163-2, G131-2 (Fig. 3) and its derivative SCR9-1 were provided by Pamela J. Hanic-Joyce. The plasmids pKRYS-L, pKRYS-S, pKRYS-ΔL and pKRYS-ΔS were generated by cloning *Bcl*I and *Sal*I digested polymerase chain reaction products from p426CCAL (for pKRYS-L and pKRYS-S) or p426CCAL-ΔNLS (for pKRYS-ΔL and pKRYS-ΔS) (from Paul B.M. Joyce) into *Bam*HI and *Sal*I digested plasmid G131-2.

Table 3: Plasmids used in this work

Plasmid	Insert	Source
p426	-	Mumberg *et al.*, 1995
pRS313	-	Sikorski and Hieter, 1989
G131-2	-	Pamela J. Hanic-Joyce

Table 3 (continued)

p426CCAL	*Arabidopsis* cDNA coding for tRNA nucleotidyltransferase starting from ATG1	Paul B.M. Joyce
p426CCAS	*Arabidopsis* cDNA coding for tRNA nucleotidyltransferase starting from ATG3	Paul B.M. Joyce
p426CCAL-ΔNLS	*Arabidopsis* cDNA coding for tRNA nucleotidyltransferase starting from ATG1 with mutations from lysine to glutamate and arginine to methionine in NLS-like sequence	Paul B.M. Joyce
p426CCAS-ΔNLS	*Arabidopsis* cDNA coding for tRNA nucleotidyltransferase starting from ATG3 with mutations from lysine to glutamate and arginine to methionine in NLS-like sequence	Paul B.M. Joyce
pBIN35S35SemGFP-ARACCA-Long	*Arabidopsis* cDNA coding for tRNA nucleotidyltransferase starting from ATG1 fused to GFP	Paul B.M. Joyce
pBIN35S35SemGFP-ARACCA-Short	*Arabidopsis* cDNA coding for tRNA nucleotidyltransferase starting from ATG3 fused to GFP	Paul B.M. Joyce
pBIN35S35SemGFP-ARACCA-Mutant Long	*Arabidopsis* cDNA coding for tRNA nucleotidyltransferase starting from ATG1 with mutations from lysine to glutamate and arginine to methionine in NLS-like sequence fused to GFP	Paul B.M. Joyce
pBIN35S35SemGFP-ARACCA-Mutant Short	*Arabidopsis* cDNA coding for tRNA nucleotidyltransferase starting from ATG3 with mutations from lysine to glutamate and arginine to methionine in NLS-like sequence fused to GFP	Paul B.M. Joyce
pBIN35S35SEmGFP	Green fluorescent protein	Dr. N. Brisson (U. de Montreal)

Table 3 (continued)

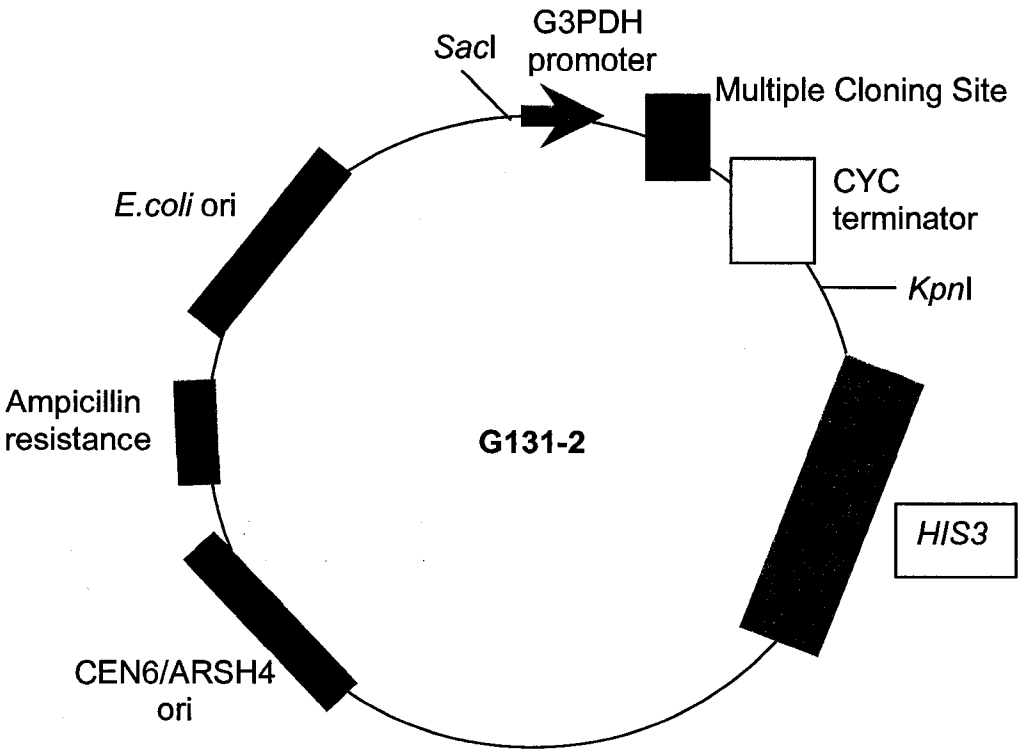
SCR9-1	*S. cerevisiae CCA1* gene with its own promoter in vector G131-2	Pamela J. Hanic-Joyce
pNLS1-S	*Arabidopsis* cDNA coding for tRNA nucleotidyltransferase starting from ATG3 with mutations in the first NLS basic domain from lysine to glutamate in vector G131-2	Shawn Karls
pNLS2-S	*Arabidopsis* cDNA coding for tRNA nucleotidyltransferase starting from ATG3 with mutations in the second NLS basic domain from lysine to glutamate and arginine to methionine	Shawn Karls
pSCCCA-L	*S. cerevisiae CCA1* gene from ATG1 of open reading frame in vector G131-2	Paul B.M. Joyce
pSCCCA-S	*S. cerevisiae CCA1* gene from ATG3 of open reading frame in vector G131-2	Paul B.M. Joyce
pKRYS-L	*Arabidopsis* cDNA coding for tRNA nucleotidyltransferase starting from ATG1 in vector G131-2	This work
pKRYS-ΔL	*Arabidopsis* cDNA coding for tRNA nucleotidyltransferase starting from ATG1 with mutations from lysine to glutamate and arginine to methionine in NLS-like sequence in vector G131-2	This work
pKRYS-S	*Arabidopsis* cDNA coding for tRNA nucleotidyltransferase starting from ATG3 in vector G131-2	This work
pKRYS-ΔS	*Arabidopsis* cDNA coding for tRNA nucleotidyltransferase starting from ATG3 with mutations from lysine to glutamate and arginine to methionine in NLS-like sequence in vector G131-2	This work

Table 3 (continued)

pKRYS-GFP-L	*Arabidopsis* cDNA coding for tRNA nucleotidyltransferase starting from ATG1 fused to GFP in vector G131-2	This work
pKRYS-GFP-ΔL	*Arabidopsis* cDNA coding for tRNA nucleotidyltransferase starting from ATG1 with mutations from lysine to glutamate and arginine to methionine in NLS-like sequence fused to GFP in vector G131-2	This work
pKRYS-GFP-S	*Arabidopsis* cDNA coding for tRNA nucleotidyltransferase starting from ATG3 fused to GFP in vector G131-2	This work
pKRYS-GFP-ΔS	*Arabidopsis* cDNA coding for tRNA nucleotidyltransferase starting from ATG3 with mutations from lysine to glutamate and arginine to methionine in NLS-like sequence fused to GFP in vector G131-2	This work
SCR9K-1	*S. cerevisiae CCA1* gene with its own promoter in vector G131-2	This work
A163-2	*S. cerevisiae CCA1* gene with a *URA3* marker	Pamela J. Hanic-Joyce

DNA coding for each Arabidopsis tRNA nucleotidyltransferase-green fluorescent protein fusion protein was moved into G131-2 as an *Xba*I fragment from the plasmids pBIN35S35SemGFP-ARACCA-Long, pBIN35S35SemGFP-ARACCA-Short, pBIN35S35SemGFP-ARACCA-Mutant-Long, and pBIN35S35SemGFP-ARACCA-Mutant-Short (generated by Paul B.M. Joyce) to produce plasmids pKRYS-GFP-L, pKRYS-GFP-S, pKRYS-GFP-ΔL and pKRYS-GFP-ΔS, respectively.

A)



B) ***Xho*I, *Sal*I, *Cla*I, *Hind*III, *Pst*I, *Eco*RI, *Eco*RV, *Sma*I, *Bam*HI, *Spe*I, *Xba*I**

Figure 3: A) Map of G131-2 containing the glyceraldehyde-3-phosphate dehydrogenase promoter, multiple cloning site (MCS), and CYC terminator of plasmid p426. The backbone is derived from vector pRS313 carrying the *HIS3* gene as a selectable marker in yeast. The MCS is cloned into the *Sac*I and *Kpn*I cut sites of plasmid pRS313. B) Available restriction sites in the multiple cloning site of G131-2.

Plasmid SCR9-1 (constructed by Pamela J. Hanic-Joyce) was digested with *Sac*I and *Sal*I to release the *S. cerevisiae CCA1* gene with its native promoter for cloning into the *Sac*I and *Sal*I sites of the vector G131-2 to generate plasmid SCR9K-1.

2.3 Polymerase Chain Reaction (PCR)

The primers and templates used to generate the PCR products are shown in Table 4. The oligonucleotide primers were purchased from BioCorp (Montreal, Quebec). To generate the inserts each PCR reaction contained ~ 10 ng of template DNA, 100 pmoles of each primer, 200 μM of dNTPs, 10X *Pfu* buffer (MBI Fermentas) and 3 units of *Pfu* polymerase (MBI Fermentas) in a final volume of 50 μl. The samples were overlaid with two drops of mineral oil to avoid evaporation at high temperatures and the reactions were performed using a Perkin Elmer DNA thermocycler. The reaction conditions used were 94°C for 4 min, followed by 35 cycles of denaturing at 94° C for 45 sec, annealing at 56°C for 1 min, and elongation at 72° C for 3 min with a final cycle at 72° C for 10 min.

Table 4: Primer Sequences

Oligo	Sequence	Purpose	Restriction site
ATG1	5'GTCGACTGATCATC TAGAATTCAACAATG ATACTAAAAACCATG 3'	5' end primer for PCR amplification of *Arabidopsis* tRNA nucleotidyltransferase cDNA from ATG1	*Bcl*I
ATG3	5'GTCGACTGATCATC TAGAATTCAACAATG ACGAATGTTGGAGAG G3'	5' end primer for PCR amplification of *Arabidopsis* tRNA nucleotidyltransferase cDNA from ATG3	*Bcl*I
ARACCA3 K	5'GAATTCGTCGACTC ACTCTATCCTTTGTCG 3'	3' end primer for PCR amplification of *Arabidopsis* tRNA nucleotidyltransferase cDNA	*Sal*I

2.4 Agarose gel electrophoresis

Agarose gels were made by dissolving agarose to a concentration of 1% in 1X TBE containing 0.5 μg/mL ethidium bromide. The desired DNA was mixed with an appropriate amount of 6X loading dye (MBI Fermentas) and loaded onto the gel. The molecular weight marker (lambda DNA digested with *Eco*RI +*Hin*dIII) was also loaded on the gel. Electrophoresis normally was carried out at 80-90 volts for approximately one hour. The DNA fragments were examined under ultraviolet light using a transilluminator.

2.5 PCR product manipulation

After each PCR amplification, an aliquot (5μl) of each PCR mix was used to confirm, by agarose gel electrophoresis (as described above), the presence of the desired PCR product. To the remainder of the sample one half volume of 7.5 M ammonium acetate and an equal volume of phenol was added. The sample was vortexed, centrifuged for 5 min at 4°C and the aqueous phase collected. To this was added an equal volume of phenol and the vortexing and centrifugation repeated. The aqueous phase was collected and extracted two times with an equal volume of ether. Two volumes of 99% ethanol were added and the sample was precipitated at -80°C for 1 hour. Samples were centrifuged at 14 000 rpm for 30 min at 4°C, the supernatant decanted, the pellet washed with 80% ethanol, desiccated for 15 min, and resuspended in 10 μl of sterile distilled water.

2.6 Restriction digestions

The enzymes used for restriction digestions were purchased from MBI Fermentas. DNA samples (0.5-2.5 μg) were incubated with the appropriate reaction buffer and two units of enzyme in a final volume of 10 μl. The mixture was incubated at the temperatures indicated by the supplier. Plasmid G131-2 was digested with the appropriate restriction enzymes and dephosphorylated with 0.5 μl of CIAP (calf intestinal alkaline phosohatase) for the final 30 minutes of digestion to prevent self-ligation. All restriction digestions were confirmed by gel electrophoresis as described above.

2.7 Phenol freeze fracture (Bewsey *et al.*, 1991)

The DNA fragments were excised from the 1% agarose gel and put into 1.5 ml Eppendorf tubes. An equal volume of phenol was added and the tubes were vigorously vortexed for a few seconds. The fragments were stored for a minimum of 30 min at -80°C and then thawed for 30 min at 37°C in a water bath. An equal volume of phenol was added and the vortexing and freeze/thaw cycle repeated. Then 100 μl of water and 1/10th volume of 3M sodium acetate was added. After vortexing, the samples were centrifuged for 10 min at 4°C and the aqueous phase was collected and extracted two times with phenol and two times with ether. Ethanol precipitation was also performed as described previously and the DNA fragments were resuspended in 10 μl sterile distilled water.

2.8 Ligation (Sambrook *et al.,* 1989)

The ligation reactions contained 6 µl of insert DNA (usually between 100 and 500 ng), 3 µl of vector (usually between 10 and 60 ng), 0.5 µl of 10 mM ATP, 2 µl 10X ligase buffer (MBI Fermentas), 0.5 µl T4 DNA ligase (5 u/µl) and distilled water to a final volume of 20 µl. Ligation reactions were incubated at 4°C overnight.

2.9 *E. coli* (XL2-Blue) transformation (Lederberg and Cohen, 1974)

Generally, 5 µl of overnight ligation mixes were added to 100 µl of XL2-Blue competent cells (prepared by Jason Arthur) and incubated on ice for 1 hour. The cells were heat shocked at 42°C for 1 min and cooled on ice. An additional 100 µl of YT medium was added and then incubated at 37°C for 1 hour, flicking the tube every 20 min. The transformed cells were plated on YT agar plates containing 50 µg/ml ampicillin and incubated at 37°C overnight. The next day at least ten colonies from each plate were patched onto a similar plate, incubated overnight at 37°C and used for a sizing gel.

2.10 Sizing gel (Barnes, 1977; Sambrook *et al.,* 1989)

The overnight patches were resuspended in 50 µl of 10 mM EDTA in an Eppendorf tube and 50 µl of 2x cracking buffer was added. After vortexing, the mixtures were incubated at 65°C for 5 min. Subsequently, 1.5 µl of 4 M KCl and 1.0 µl of 0.4% bromophenol blue were added and the samples vortexed and incubated on ice for 5 min. The mixtures were centrifuged in an Eppendorf microfuge at 14 000 rpm for 5 min at

room temperature and 20 µl of the supernatant was loaded onto a 0.7% agarose gel. Gel electrophoresis was performed as described above. A control of vector without insert was also electrophoresed on the gel for comparative purposes.

2.11 Plasmid preparation (Berghammer and Auer, 1993)

E. coli cells carrying the plasmids of interest were grown overnight at 37°C with shaking in 5 ml of YT containing ampicillin (50 µg/ml). The cultures were collected in Eppendorf tubes and centrifuged in an Eppendorf microfuge at 14 000 rpm for 5 min at room temperature. The pellets were then resuspended in 50 µl easy prep buffer, shaken for 10 min at room temperature, boiled for 60 sec, put on ice for 60 sec, and then centrifuged in an Eppendorf microfuge at 14 000 rpm for 15 min at room temperature. The supernatants were collected and subjected to two phenol extractions and two ether extractions as described before. The DNA was precipitated and washed with ethanol also as described before and the DNA samples were resuspended in 10 µl sterile distilled water.

2.12 Preparation of competent yeast cells (Schiestl and Gietz, 1989)

Single colonies of SCDT-6, YPH500, or E189F were inoculated into 5 ml YPD liquid medium and incubated in a water bath until stationary phase (48 hours for SCDT-6 and YPH500 at 30°C, and 5 days for the temperature-sensitive E189F at 22°C). A 1 ml aliquot of the stationary phase culture was used to inoculate 300 ml of YPD with

incubation at the appropriate temperature until an OD_{640} of 0.7 was obtained. Using a JA-14 rotor, the culture was centrifuged at 5000 rpm for 10 min at room temperature in a Beckman J2HS centrifuge. The pellet was washed with 150 ml of sterile distilled water twice and resuspended with sterile distilled water to reach a final volume of 2.7 ml. Then 300 µl of 1 M LiAc (pH 7.5) was added and the mixture was incubated at room temperature for 20 min. The cells were mixed by inversion, collected in Eppendorf tubes (500 µl), and centrifuged at 14 000 rpm for 1 min. The pellets were resuspended with 0.1 M LiAc (pH 7.5) to reach a total volume of 250 µl. Finally, 150 µl of 50% glycerol was added, mixed by inverting and the Eppendorf tubes were stored at -80° C.

2.13 Yeast transformation (Akada, 2000)

Aliquots (50 µl) of the SCDT-6, YPH500, or E189F competent cells prepared as above were centrifuged at 14 000 rpm for 1 min at room temperature in an Eppendorf centrifuge. The supernatant was discarded and the pellet was layered with 10 µl (5-10 µg) of plasmid DNA, 12.5 µl of herring sperm DNA (7.4 mg/ml) provided by Pamela J. Hanic-Joyce, and 500 µl of PLAG solution. The pellet was resuspended with a pipette and the mixture was incubated at 42°C for approximately 90 min (only 15 min for the temperature-sensitive strain E189F) and centrifuged at 14 000 rpm in an Eppendorf microfuge for 15 min at room temperature. The resulting pellet was resuspended with 50 µl of sterile distilled water and plated onto synthetic complete medium lacking the appropriate amino acids. The plates were incubated at 30°C for two days for the strains SCDT-6 and YPH500 and at 22°C for ~ 5 days for strain E189F.

2.14 Plasmid shuffling

SCDT-6 cells contained a rescue plasmid that carried a *URA3* selectable marker and a functional *S. cerevisiae CCA1* gene. Plasmid G131-2 with or without insert and carrying the *HIS3* selectable marker was introduced by transformation as described previously and the cells were grown at 30°C on synthetic complete medium lacking the appropriate amino acids (SC-ura-his) for two to three days. Cells were patched on SC-ura-his plates, allowed to grow and replica plated on YPD. After growth on YPD for two days at 30°C they were replica plated onto SC-his plate, allowed to grow, and finally replica plated on a plate containing SC+FOA and left at 30°C for three days.

2.15 Testing the ability to complement a temperature-sensitive mutation

E189F cells carrying the plasmids of interest were left to grow at the permissive temperature (22°C) on SC-his plates and then replica plated onto the same medium and shifted to the non-permissive temperature (37°C). At the same time they were replica plated to plates containing the same medium and allowed to grow at the permissive temperature.

2.16 Testing the ability to grow on a non-fermentable carbon source

Cells of strain SCDT-6 carrying plasmids of interest that grew on FOA were patched onto SC-His plates, allowed to grow and then replica plated onto YPD and YPG plates. Colony growth on plates was recorded after two days at 30°C.

2.17 Fluorescence microscopy

S. cerevisiae YPH500 cells were stained using DAPI (4', 6'- diamidino-2-phenylindole dihydrochloride) as follows (Moreno *et al.*, 1991): 900 µl of exponentially growing cells (OD_{640} = 0.7-1) were fixed with 100 µl of 37% formaldehyde for 30 min at the same temperature that the cells were growing. The cells were then centrifuged for 30 sec at room temperature, washed in 1ml of sterile PBS, washed in 1 ml PBS containing 1% Triton X-100, and resuspended in 100 µl of PBS.

A microscope coverslip was coated with 1 mg/ml of poly L-lysine. The cells were applied to the coverslip as a monolayer by placing about 15 µl of the cell suspension on the coverslip, then removing most of the cells such that only a thin film of the cells remained behind. The coverslip was left to dry at room temperature and dropped, face down, onto 2 µl of DAPI mounting solution on a microscope slide. Finally, the edges of the coverslip were sealed with nail polish and examined under the Zeiss fluorescence microscope at 100X oil magnification.

To visualize DAPI, the UV-G 365 filter cube set was used (UV Filter Cube set #487902: Exciter Filter: G 365, Beam Splitter: FT 395, Barrier Filter: LP 420). To visualize GFP, the blue 450-490 SB filter cube set was used (Blue Filter Cube set #487910: Exciter Filter: BP 450-490, Beam Splitter: FT 510, Barrier Filter: BP 515-565). Images were collected using a SPOT Insight 2 megapixel color mosaïc digital camera and the Spot advanced version 4.0.9 software (jpeg files) with the image type: brightfield-transmitted light, and manual exposure as image settings. To obtain a clearer view, images were manipulated using the software Adobe photoshop CS version 8.0.

2.18 Activity assay for tRNA nucleotidyltransferase

2.18.1 Preparation of crude extracts

Crude cell extracts for enzyme assays were prepared using the procedure of Najarian *et al.* (1986). Mid-log phase E189F cells (OD_{640} = 0.7-1) carrying plasmids of interest were centrifuged at 5000 rpm for 15 minutes at 4°C in a Beckman J2HS centrifuge using a JA-20 rotor. Pellets were weighed and resuspended in 1 ml enzyme assay buffer for every 0.5 g of cells. An equal volume of glass beads (to the buffer) was then added, and the samples were vortexed for 2 minutes, centrifuged in an Eppendorf microfuge at 14 000 rpm at 4°C for 10 minutes, and the supernatant transferred to new Eppendorf tubes. The concentration of the proteins was determined with the Bradford Assay dye (Bio-rad) using bovine serum albumin as the standard.

2.18.2 Enzyme assay

The enzyme activity assay was based on the procedure of Cudny *et al.* (1978) and measured the incorporation of α^{32}P-CMP into tRNAs lacking the 3' CCA sequence. Reactions were carried out in duplicate in glass tubes in final volumes of 100 µl assay mix with or without 20 µM tRNA. The tRNA was pre-treated with snake venom phosphodiesterase (by Jason Arthur) to remove intact CCA sequences. Crude extract containing 100 µg of total protein, α^{32}P-CTP, ATP, and tRNA were mixed with the appropriate buffer and the assay was performed for 2 minutes at room temperature and stopped by adding the same volume of 2M HCl with incubation on ice for 20 minutes.

The reaction mixtures were then filtered through GF/C Whatmann glass fiber filters to collect tRNAs containing α^{32}P-CMP. Filters were washed 20 times with 10 ml 1M HCl and finally once with 10 ml 99% ethanol. The filters were air dried and placed in scintillation vials with 5 ml of biodegradable counting scintillant (Amersham International Ltd.). Activity was measured in an LKB WALLAC 1217 RACKBETA scintillation counter, program A15 (to measure ^{32}P). All reactions were carried out in duplicate and every experiment was carried out more than once.

3. RESULTS

3.1 Polymerase chain reaction (PCR)

I began my studies of the potential nuclear localization signal of Arabidopsis tRNA nucleotidyltransferase by PCR amplifying cDNAs coding for four versions of the tRNA nucleotidyltransferase starting at ATG1 or ATG3 with wild-type or modified potential nuclear localization signal. PCR products of expected sizes were generated from all four of the Arabidopsis clones (Figure 4). Note that the products generated from the primer pair containing oligonucleotide ATG1 (coding for amino acid 1) are approximately 200 bp larger than those produced from the primer pair containing oligonucleotide ATG3 (coding for amino acid 68). This is in good agreement with the additional sixty-seven amino acids that should be found in the protein generated from this construct.

3.2 Cloning the tRNA nucleotidyltransferase PCR products into G131-2

For further analysis, the wild-type and variant tRNA nucleotidyltransferase were introduced into the yeast expression vector G131-2 under the control of the yeast glyceraldehydes-3-phosphate dehydrogenase promoter. The PCR fragments were digested with *Bcl*I and *Sal*I and cloned into the G131-2 vector (digested with *Bam*HI and *Sal*I) to generate pKRYS-L, pKRYS-S, pKRYS-ΔL, and pKRYS-ΔS. *Eco*RI restriction

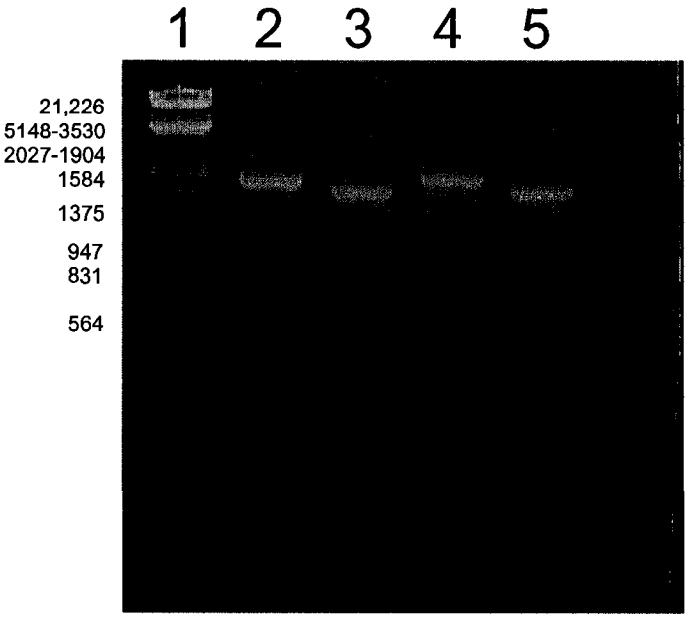


Figure 4: Agarose gel showing products generated from polymerase chain reaction (PCR) amplification of cDNAs coding for *Arabidopsis* tRNA nucleotidyltransferase. Lane 1: Molecular weight markers (λ DNA digested with *Hin*dIII and *Eco*RI). Lane 2: PCR product generated from the wild-type cDNA using the ATG1 5'-oligo. Lane 3: PCR product generated from the wild-type cDNA using the ATG3 5'-oligo. Lane 4: PCR product generated from the mutant cDNA using the ATG1 5'-oligo. Lane 5: PCR product generated from the mutant cDNA using the ATG3 5'-oligo. All PCR products were generated using the same 3'-oligo (ARACCA3K).

fragments of ~1400 bp and ~ 1200 bp confirmed the presence of single inserts for the plasmids pKRYS-L (or pKRYS-ΔL) and pKRYS-S (or pKRYS-ΔS), respectively. After cloning the various tRNA nucleotidyltransferase-GFP fusion constructs into G131-2 as an *Xba*I fragment, the plasmids pKRYS-GFP-L, pKRYS-GFP-S, pKRYS-GFP-ΔL and pKRYS-GFP-ΔS were digested with *Xba*I to confirm the presence of insert and with *Bam*HI to confirm the orientation of the insert (data not shown). *Xba*I digestion released the entire insert whereas *Bam*HI digestion released a ~ 1700 bp fragment if the orientation was correct and a ~ 900 bp fragment if the orientation was reversed. Plasmid SCR9K-1 containing the *S. cerevisiae CCA1* gene with its native promoter in vector

G131-2 was digested with *Sac*I and *Sal*I to show the presence of insert. Plasmids showing the correct inserts in the correct orientation were transformed into yeast.

3.3 Plasmid shuffling

The yeast strain SCDT-6 with its chromosomal *CCA1* gene replaced by *TRP1* and carrying plasmid SCR9-1 (which contains the *S. cerevisiae CCA1* gene and the *URA3* gene) was transformed with plasmid G131-2, pKRYS-L, pKRYS-S, pKRYS-ΔL, pKRYS-ΔS, pKRYS-GFP-L, pKRYS-GFP-S, pKRYS-GFP-ΔL, pKRYS-GFP-ΔS or SCR9K-1 to determine which genes could complement the loss of the *S . cerevisiae CCA1* gene. Single colonies were selected from SC-ura-his plates and patched onto SC-ura-his plates. These patches were replica plated onto YPD, allowed to grow, replica plated onto SC-his plates, allowed to grow, and finally replica plated from SC-his onto SC plates containing FOA (5'-fluoroorotic acid). Cells bearing plasmids pKRYS-L, pKRYS-S, pKRYS-GFP-L, pKRYS-GFP-S or SCR9K-1 grew well on FOA while cells carrying plasmids pKRYS-ΔL, pKRYS-ΔS, pKRYS-GFP-ΔL, pKRYS-GFP-ΔS or G131-2 showed little or no growth on FOA (Figure 5).

While the cells carrying plasmids with the yeast *CCA1* gene or the wild-type Arabidopsis cDNAs coding for the tRNA nucleotidyltransferase grew in the presence of FOA (indicating that these constructs could complement the loss of the *S. cerevisiae CCA1* gene), none of the mutant Arabidopsis cDNAs could complement this defect.

To further support these observations and to see if the remaining yeast tRNA nucleotidyltransferase protein found in the ts mutant could exert any effect on the

function of the Arabidopsis enzyme, the same plasmids were transformed into the temperature-sensitive yeast strain E189F which was replica plated onto SC-his plates at both the permissive (22°C) and non-permissive (37°C) temperatures (Figure 6).

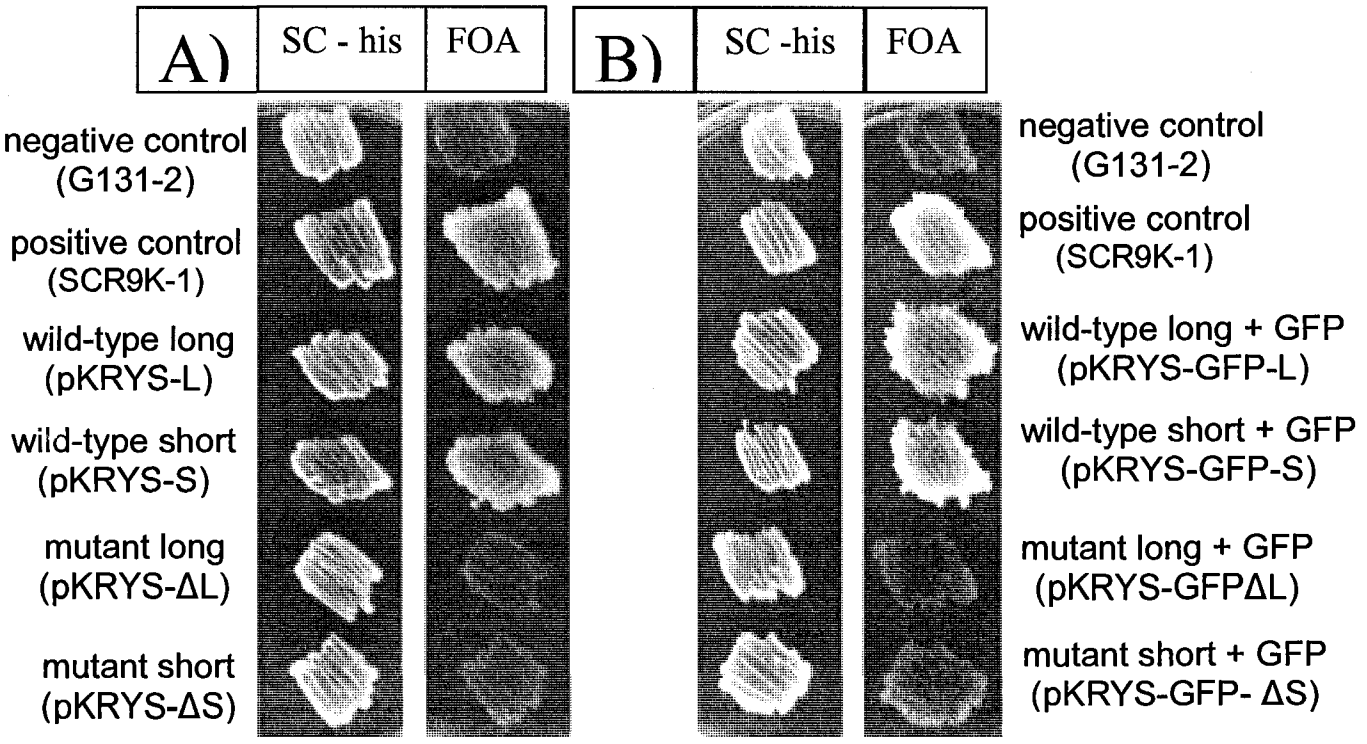


Figure 5: Plasmid shuffling: Cells were patched onto SC-ura-his plates, replica plated twice onto YPD plates and allowed to grow. The resulting colonies were replica plated onto SC -his plates, allowed to grow and then replica plated onto SC plates containing FOA. The examples presented show growth after 3-4 days at 30°C. A) Arabidopsis cDNAs alone. B) Arabidopsis cDNAs fused to GFP.

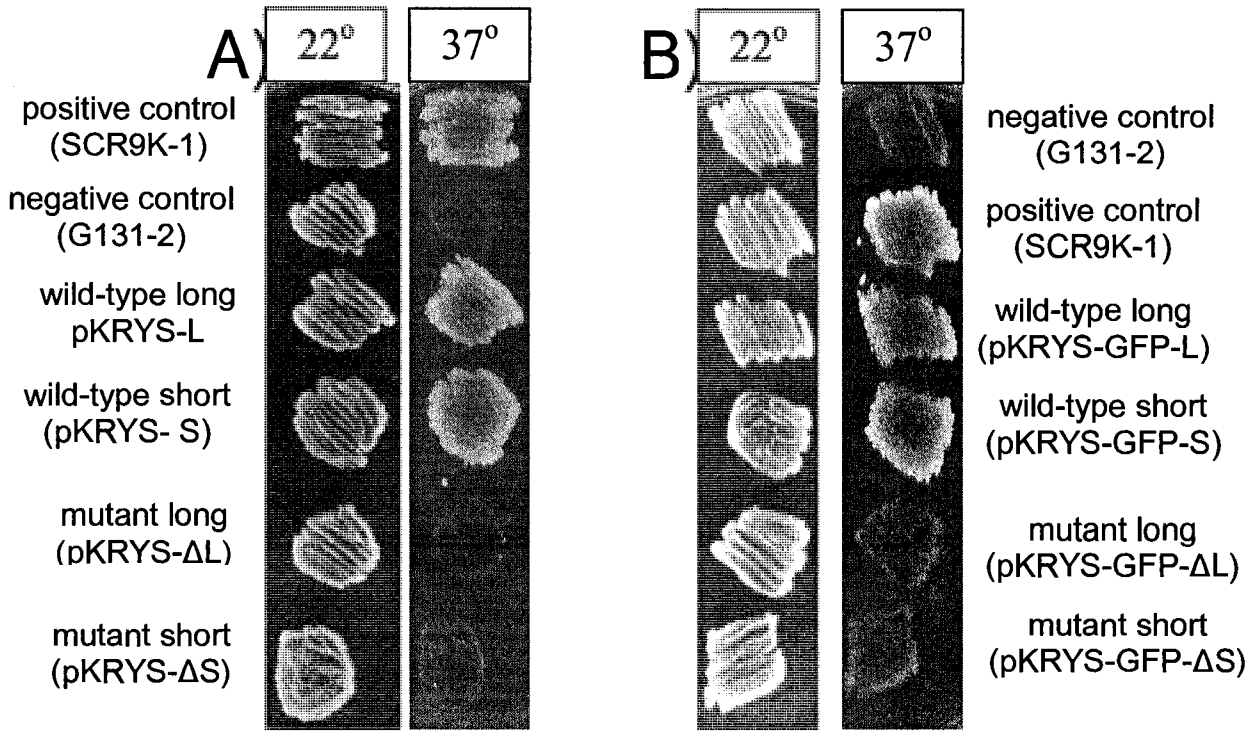


Figure 6: Temperature-sensitivity of cells carrying plasmids of interest. Cells were patched onto SC-his plates at the permissive temperature (22°C) and then replica plated to SC-his plates and placed at the permissive (22°C) and non-permissive (37°C) temperatures. A) Arabidopsis cDNAs alone. B) Arabidopsis cDNAs fused to GFP.

Taken together these data indicate that while the Arabidopsis wild-type tRNA nucleotidyltransferase is able to replace the yeast enzyme, the variant enzymes did not. The reasons for this remain to be determined but could include loss of stability, incorrect localization, or loss of activity.

To address the role of the amino-terminal sequences in mitochondrial function of the Arabidopsis tRNA nucleotidyltransferase in yeast, the Arabidopsis cDNAs were used to try to complement the mitochondrial defect resulting from the loss of the *S. cerevisiae CCA1* gene. In this case the cells showing growth on FOA were patched onto SC-His plates, allowed to grow, and then replica plated onto YPD and YPG (Figure 7).

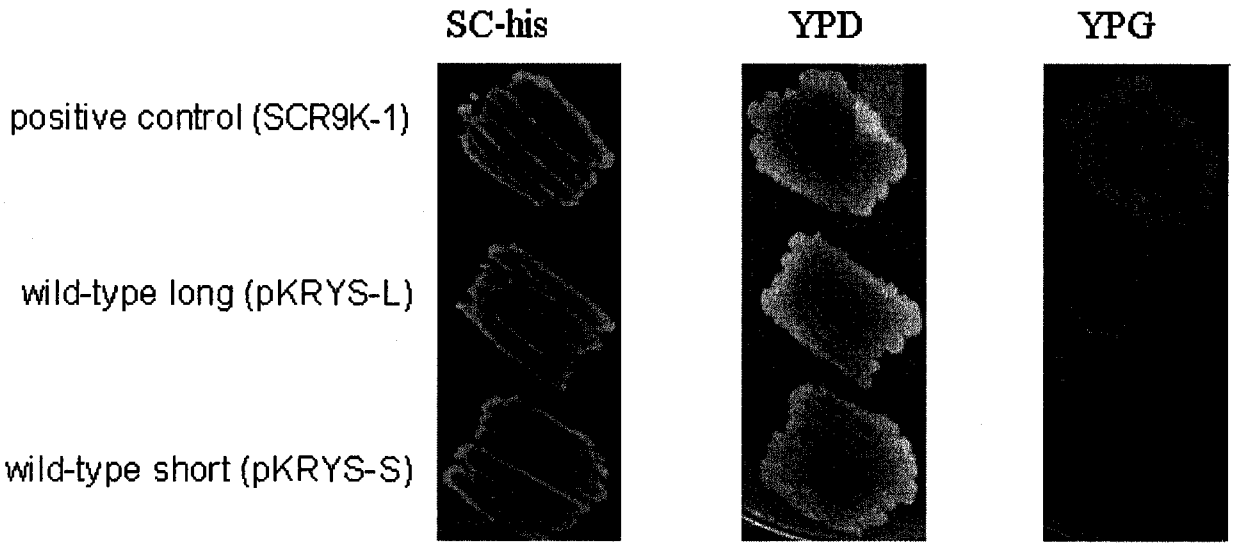


Figure 7: Cell viability on YPD and YPG: The cells on FOA were patched on to SC-his plates and replica plated onto YPD and YPG.

In each case cells containing the Arabidopsis tRNA nucleotidyltransferase were unable to grow on the non-fermentable carbon source suggesting that this enzyme does not function in yeast mitochondria. Given that the enzyme functions in the cytosol, as indicated by growth on YPD (Figure7), this suggests that these amino terminal sequences do not function as a mitochondrial targeting signal in yeast.

3.4 Enzyme activity assay

To see if the variant Arabidopsis tRNA nucleotidyltransferase was still active, enzyme activity assays were carried out using the temperature-sensitive strain carrying the plasmids of interest. When these activity assays were carried out at 22°C under the standard assay conditions in extracts from E189F, the extracts containing plasmids

expressing the Arabidopsis proteins showed activity comparable to extracts containing plasmids expressing the yeast enzyme whereas the extracts prepared from strains containing plasmids expressing the variant Arabidopsis proteins that failed to complement showed similar activity to strains carrying the empty G131-2 plasmid alone (Figure 8). (The raw data indicating the amount of radioactivity incorporated are shown in Appendix A).

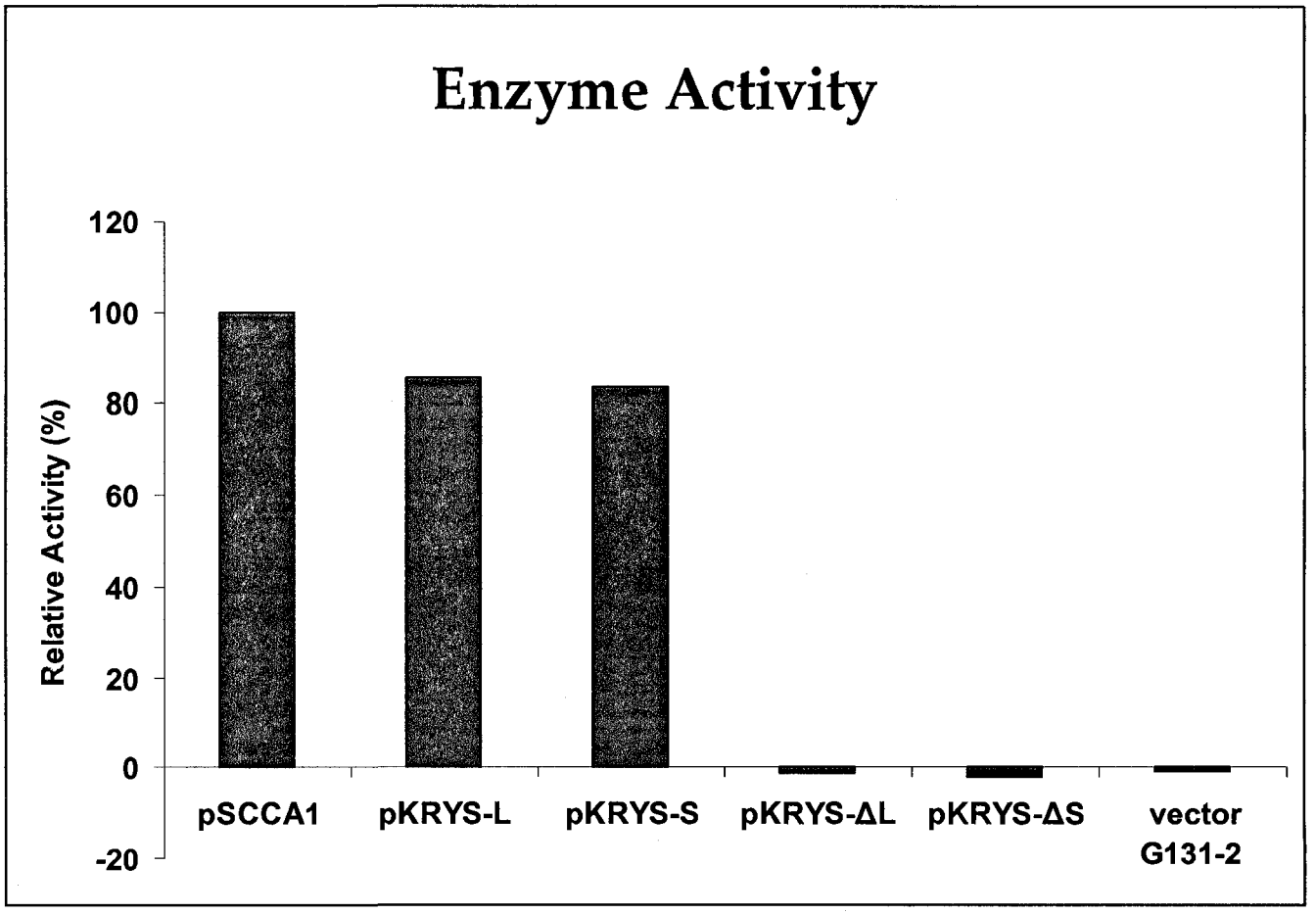


Figure 8: tRNA nucleotidyltransferase activity seen in crude extracts from strain E189F. The cells carried the following plasmids: 1) pSCCA1 (*S. cerevisiae CCA1* gene in G131-2, positive control); 2) pKRYS-L (*Arabidopsis* cDNA wild-type long); 3) pKRYS-S (*Arabidopsis* cDNA wild-type short); 4) pKRYS-ΔL (*Arabidopsis* cDNA mutant long); 5) pKRYS-ΔS (*Arabidopsis* cDNA mutant short); 6) Vector G131-2 alone (negative control).

These results are in good agreement with the *in vivo* results (Figures 5 and 6). The strains that are viable at the non-permissive temperature show enzyme activity in extracts while those that die at the non-permissive temperature show little or no activity in their extracts. Although this result suggests that the variant enzymes are being generated but are inactive it is also possible that the variant proteins are unstable and rapidly degraded. To address this possibility, GFP fusion proteins were constructed.

3.5 Expression of tRNA nucleotidyltransferase-GFP fusion proteins in *S. cerevisiae*

To see if Arabidopsis tRNA nucleotidyltransferase fusion proteins are being generated, accumulated, and targeted in yeast cells, the plasmids G131-2, pKRYS-GFP-L, pKRYS-GFP-S, pKRYS-GFP-ΔL, pKRYS-GFP-ΔS, pSCCA-L or pSCCA-S were transformed into yeast strain YPH500 as described and the transformants viewed on a Zeiss fluorescence microscope at 100X oil magnification. When no GFP was expressed in yeast no fluorescence was seen (Figure 9 A I). In contrast, when the plasmid containing GFP alone was expressed in yeast cells a generally equal distribution of fluorescence was seen throughout each cell (Figure 9 B I). A similar distribution of fluorescence was seen in cells expressing yeast tRNA nucleotidyltransferase fused to GFP (Figure 9 C I and D I). These results suggest that at steady state most of the native yeast tRNA nucleotidyltransferase is found in the cytosol and argue against the presence of an efficient nuclear localization signal on this enzyme. This observation supports previous findings using indirect immunofluorescence to measure yeast tRNA nucleotidyltransferase distribution (Wolfe *et al.*, 1996). When cells carrying either

pKRYS-GFP-L or pKRYS-GFP-S and expressing the long (Figure 9 E) or short (Figure 9 F) form of the Arabidopsis tRNA nucleotidyltransferase, respectively, fused to GFP were analyzed they also showed detectable fluorescence. Both of these constructs showed a similar distribution of fluorescence with some apparent enrichment in the nucleus as compared to the cytosol (Figure 9 E I and F I). On the other hand, fluorescence in cells carrying pKRYS-GFP-ΔL, and pKRYS-GFP-ΔS and expressing the modified Arabidopsis proteins was clearly more intense in the nucleus than in the cytosol (Figures 9 G I and H I).

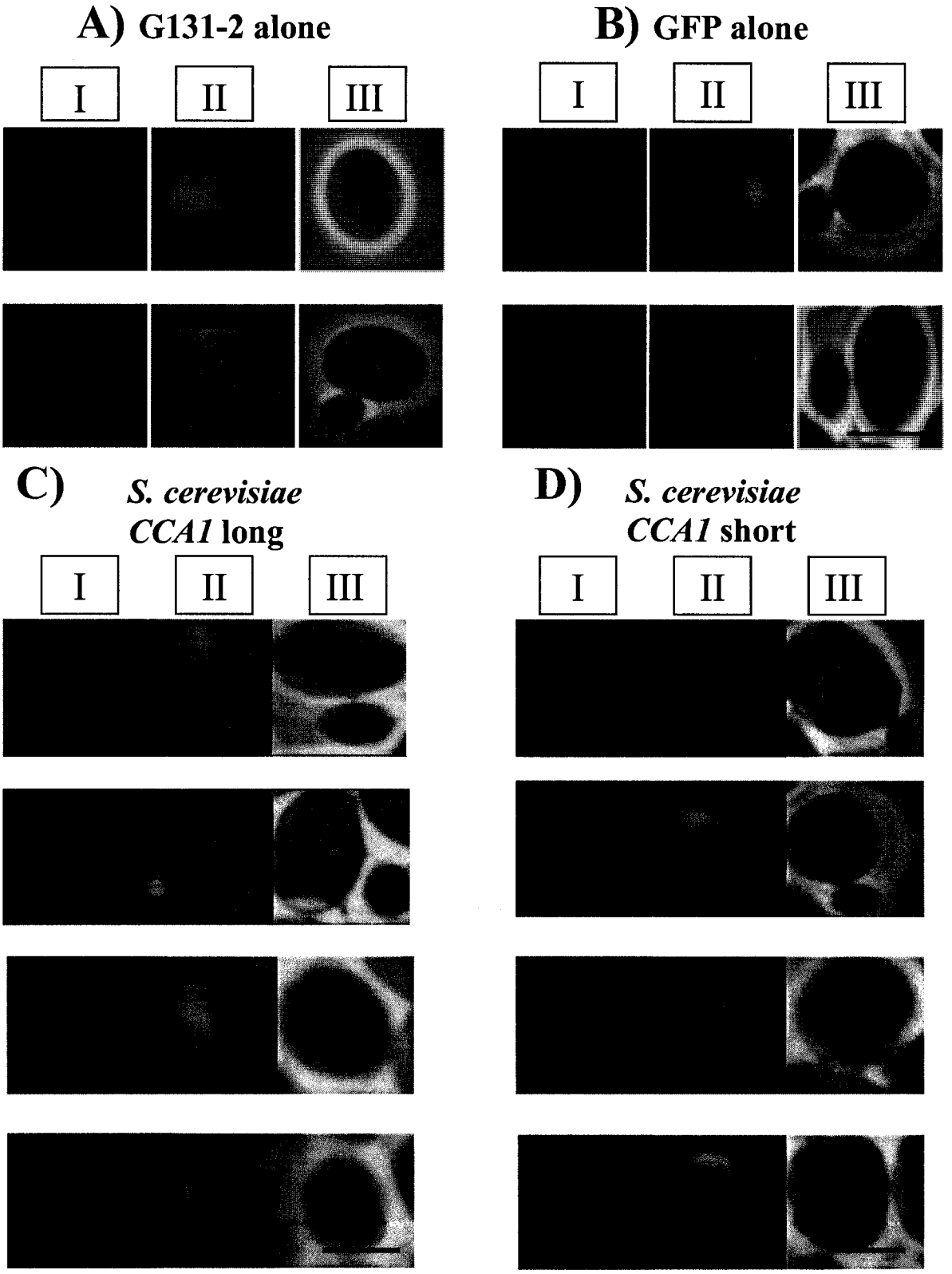


Figure 9: Fluorescence distribution from tRNA nucleotidyltransferase-GFP fusion proteins in yeast. A) Plasmid G131-2 alone. B) Green fluorescent protein (GFP) alone. C) *S. cerevisiae CCA1* gene starting at ATG1. D) *S. cerevisiae CCA1* gene starting at ATG3.

Three views of each cell are shown: I) GFP fluorescence, II) DAPI fluorescence, III) phase contrast. Multiple panels are provided in each example to show a minimum of two cells containing the same plasmid. Scale bar = 10 μm.

One point to take away from these results is that the steady-state level of the modified tRNA nucleotidyltransferase-GFP fusion proteins seems to be sufficient to suggest that the loss of activity is not simply due to a more rapid breakdown of the modified protein. The second point is that the wild-type Arabidopsis proteins show a similar level of fluorescence as the yeast tRNA nucleotidyltransferase-GFP fusion protein with perhaps a slight increase in nuclear localization. The final point to note is that the distribution of the modified Arabidopsis proteins is different from that of the wild-type Arabidopsis proteins.

DAPI fluorescence was used to normalize the various GFP panels and to show that the difference in degrees of intensities is the result of actual fusion-protein concentration changes and not exposure time variances. The intensity of the DAPI is the same in virtually all the panels and defines the nuclear location in each cell. Furthermore, the fact that the DAPI signal remained constant in the presence of all plasmids suggests that the cell's nuclei were relatively healthy and viable despite the presence of additional plasmids and foreign protein (Figure 9 panels A-H, II). Similar results were obtained when strains SCDT-6 or E189F were used (data not shown).

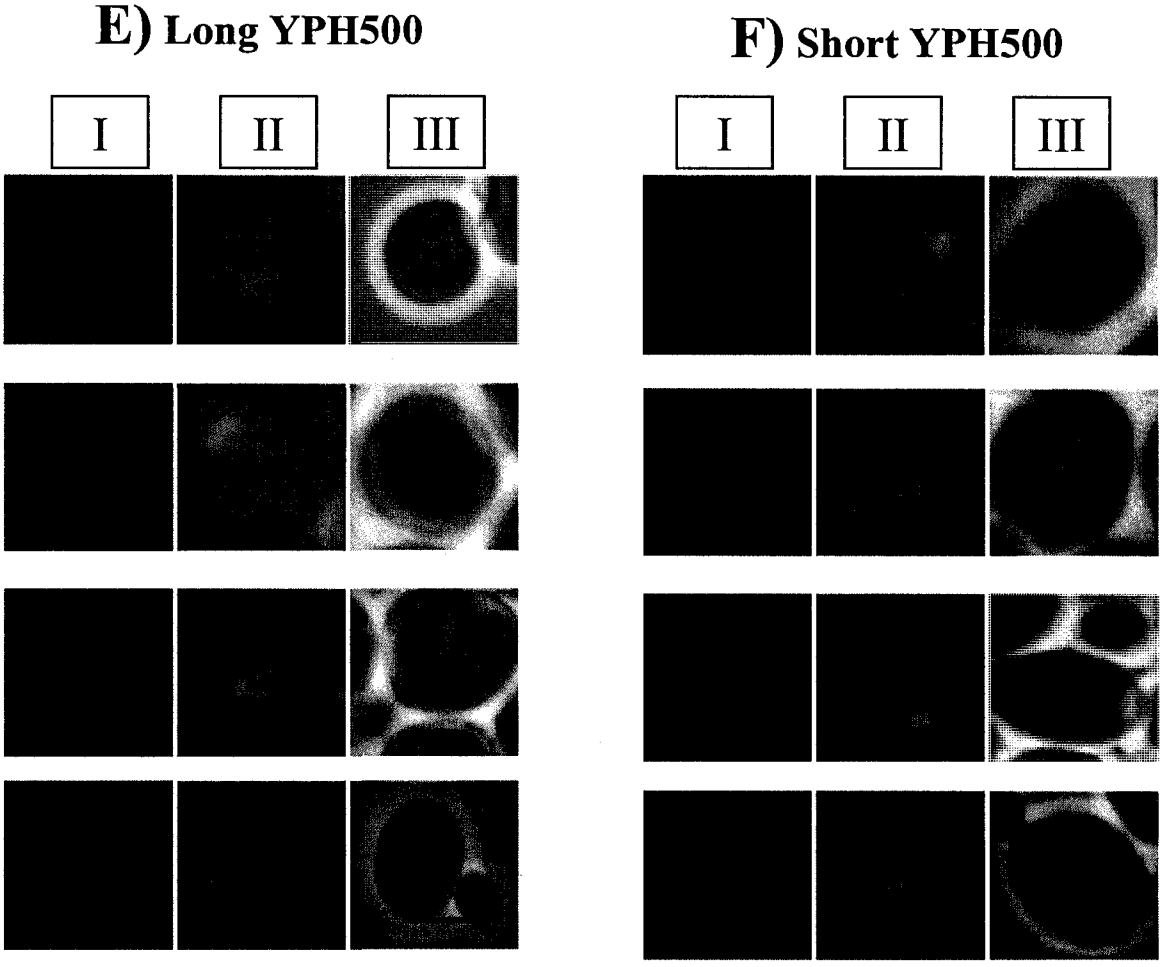


Figure 9 (continued): E) wild-type long *Arabidopsis* cDNA coding for tRNA nucleotidyltransferase (pKRYS-GFP-L). F) wild-type short *Arabidopsis* cDNA coding for tRNA nucleotidyltransferase (pKRYS-GFP-S). Three views of each cell are shown: I) GFP fluorescence, II) DAPI fluorescence, III) phase contrast. Multiple panels are provided in each example to show a minimum of two cells containing the same plasmid. Scale bar = 10 μm.

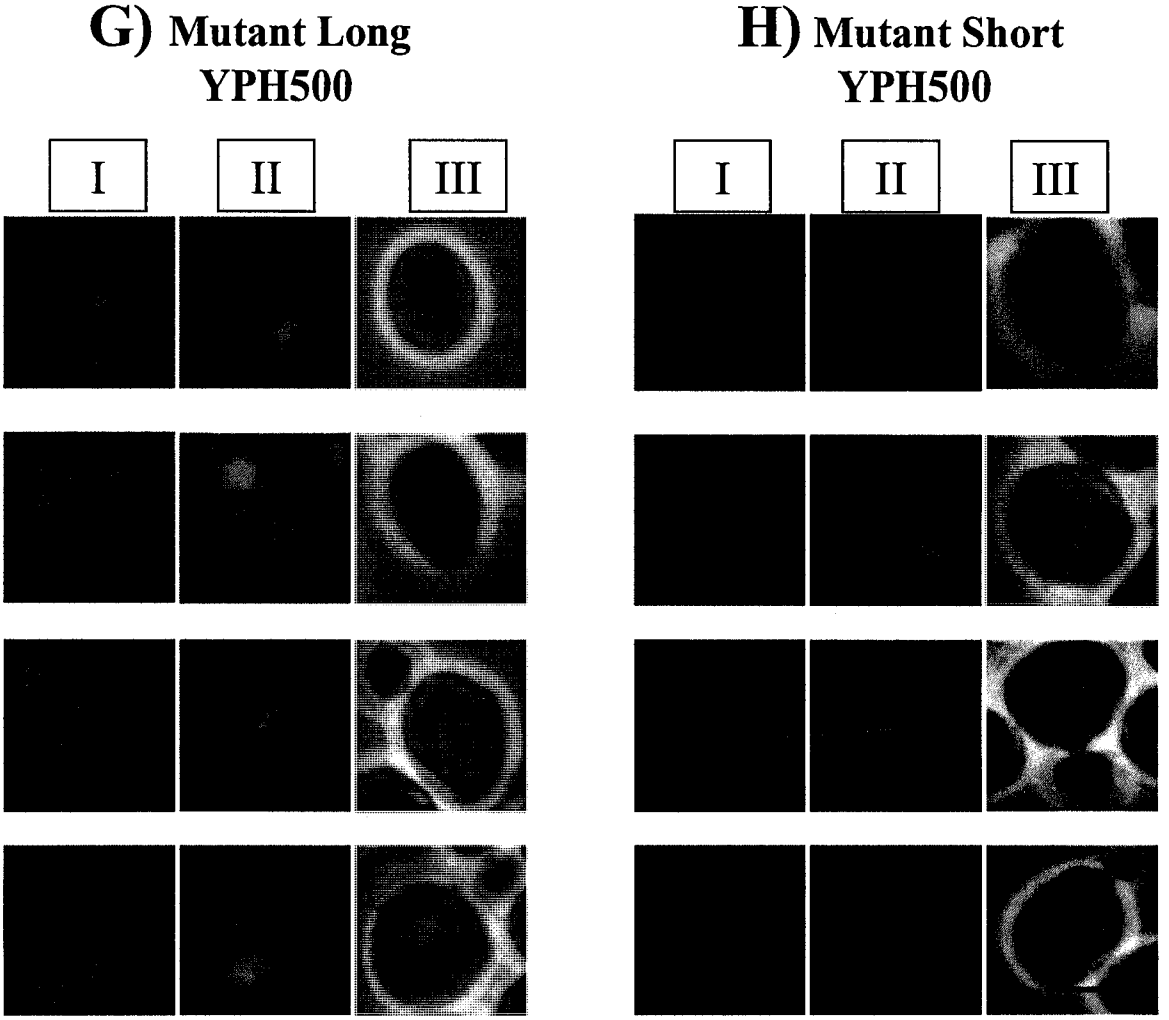


Figure 9 (continued): G) mutant long *Arabidopsis* cDNA coding for tRNA nucleotidyltransferase (pKRYS-GFP-ΔL). H) mutant short *Arabidopsis* cDNA coding for tRNA nucleotidyltransferase (pKRYS-GFP-ΔS). Three views of each cell are shown: I) GFP fluorescence, II) DAPI fluorescence, III) phase contrast. Multiple panels are provided in each example to show a minimum of two cells containing the same plasmid. Scale bar = 10 μm.

3.6 Role of bipartite targeting signal

Since combining mutations in both positive clusters of amino acids in the potential bipartite targeting signal resulted in the production of proteins that were non-functional *in vivo* (Figures 5 and 6) and *in vitro* (Figure 8) it became important to see what effect modifying only one set of these amino acids in the targeting signal would

have. By checking for growth of temperature-sensitive cells carrying plasmids expressing tRNA nucleotidyltransferase with modifications to either set of these basic amino acids at the non-permissive temperature it became apparent that altering either set of amino acids was sufficient to inactivate the protein (Figure 10).

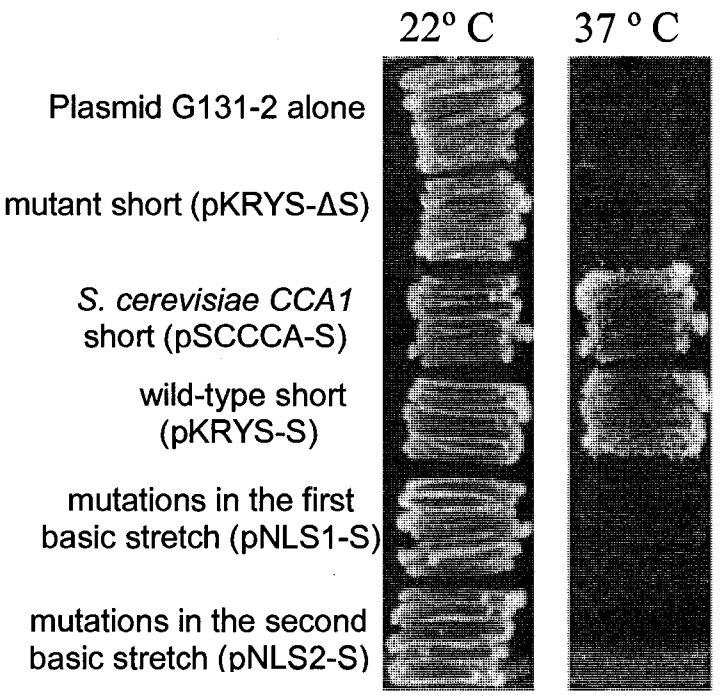


Figure 10: Temperature-sensitivity of cells carrying plasmids of interest. Cells were patched onto SC-his plates at the permissive temperature (22°C) and then replica plated to SC-his plates and placed at the permissive (22°C) and non-permissive (37°C) temperatures.

4. DISCUSSION

4.1 A potential nuclear localization signal identified in the Arabidopsis tRNA nucleotidyltransferase

In this study we set out to characterize a potential nuclear localization signal in the Arabidopsis tRNA nucleotidyltransferase. We used the expression of the wild-type and variant Arabidopsis proteins in *S. cerevisiae* to examine the possible role of this sequence in protein function and localization. To identify a sequence that could play a role in nuclear targeting, we first analyzed ADEPT (Stanford *et al.*, 2000) regions of the Arabidopsis protein. ADEPTs are defined as additional domains for eukaryotic protein targeting and are regions found in eukaryotic proteins but absent from their prokaryotic homologues. We concentrated on domains found in the Arabidopsis protein but absent from its *E. coli* homologue (highlighted in Figure 2). As these regions of the protein show no corresponding regions in the *E. coli* enzyme we argued that they would not be required for enzyme function, but might play a eukaryotic specific role, perhaps in protein targeting. In one of the largest ADEPTs in the Arabidopsis enzyme we identified a sequence (KDTKGKSIPVVNHIFKFSMKRK) that had the organization and amino acid character of a bipartite nuclear localization signal (Dingwall *et al.*, 1988). It is characterized by two clusters of basic amino acids (lysines and arginine) separated by a spacer region of about 10 amino acids (13 in this case). This specific type of NLS is common in eukaryotes and mediates nuclear import through the importin-α pathway (Kalderon *et al.*, 1984). Importin-α itself contains its own bipartite NLS, crucial for importin-β binding and consequent nuclear import (Kalderon *et al.*, 1984; Dingwall *et al.*, 1988).

That this sequence may play a role in the structure or function of tRNA nucleotidyltransferase, at least in plants, came from the observation that it was conserved both in character and location in the dicotyledonous plants *Lupinus albus* (U15930) and tomato (BT013425) and in the monocotyledonous plant rice (NM_001072807) (Figure 11). Given these observations and the absence of any obvious nuclear localization signals, apart from some short stretches of lysine residues, in the other eukaryotic tRNA nucleotidyltransferases we identified this as the best candidate for a nuclear localization signal in the Arabidopsis tRNA nucleotidyltransferase. We, therefore, expressed the Arabidopsis tRNA nucleotidyltransferase containing this sequence or a variant form of this sequence in *S. cerevisiae* to study the activity and localization of these proteins.

```
S.cerev     ENCQDMDKINHVYNDNILNSHLKSFIELYPMFLEKLPILREKIGRSP-GFQQNFILSAIL
C.glabrata KNWPQTKDVEDIYKKGIFNHHLKNFIHHYKDFLSRYLKLRQAIETKDKSFQQNFLLASIL
K.lactis   ---ESSPEIESIYEN--LDQHLKSVVETIPKLLKSHTTFASVFPGMQEPLILSLVLSGFK
lupin      SQLDISWNLIHLLGKTTFTDEQRRLTLYAAMFLPLRNTIYREKKAKKVPVVNYIFRESLK
Arabidopsi SYLEAMWSLLKTPRPGKFSGEQRRLALYAAMFLPFRKTVYKDTKGKSIPVVNHIFKFSMK
Tomato       LGSAWELLNLIGRSSFSDDQRRLCLYAALFLPFRETIYRDNKAKKIPVVNYIFRNSLK
Rice       LAHSIGCSVFSGGSDSKSQDEHRRLCFYSALFTPVRNTIYLDKKSKKIPVTNYIIRDSLK
human      ------------------LEEFDKVSKNVDGFSPKPVTLLASLFKVQDDVTKLDLRLKIA

S.cerev     SPMANLQIIGNPKKKINNLVSVTESIVKEGLKLSKNDAAVIAKTVDSICSYEEILAKFAD
C.glabrata IPMADLKIIALPKKKLNNTLPVSESIVREGLKFNKASSIVVARCVENIAAYNSMVEKYLQ
K.lactis   G------LKGPDPAKPKNSIPLAGVITKEGLNFPNTQVDNVIACVESEDSYHNLVKNGKS
lupin      RKAKDPETVLDLHRASNKFLSLIPCLVSN--EDVQIVGHDWMTELID----------VP
Arabidopsi RKTSDAETVMNIHQTTERFRSLIPSLEVK-KDVELDELTWAADILEHWKSITLNDPVIP
tomato     LKASDAEIVMSLHTVTRKFVTLISLLISKDDIQVLEVDWER-DTIE----------VPIA
Rice       LKASDADTVVNVHAASEKFAELINLLESNENLTTVKEKLD--DEYLE---------IPTD
human      KEEKNLGLFIVKNRKDLIKATDSSDPLKP----------------------------
```

Figure 11: ClustalW alignment of eight eukaryotic tRNA nucleotidyltransferases. Only the regions corresponding to those flanking the apparent nuclear localization signal in Arabidopsis are shown. The nuclear localization-like sequences in Arabidopsis, lupin, tomato and rice are underlined and all of the basic residues are shown in bold.

4.2 This sequence was studied in a heterologous system

In this study we chose to examine the role of these amino acids in the Arabidopsis protein in a heterologous yeast system while, simultaneously, others (Schmidt von Braun *et al.*, accepted) were studying the role of these amino acids *in planta*. There are practical advantages to using yeast as an experimental model. For example, the relative ease with which yeast cells can be transformed and foreign proteins expressed and characterized in them. In addition, we had on hand both knockout and conditionally defective yeast strains to help us explore the role of this motif in enzyme function. Moreover, given the conservation of nuclear localization signals (Lanning *et al.*, 2004; Tzifra and Citovsky, 2005; Fries *et al.*, 2007) and the nuclear import process in different organisms (Görlich and Mattaj, 1996; Siomi *et al.*, 1998; Tzifra and Citovsky 2005) we anticipated that results obtained in yeast could be related directly to those obtained *in planta*. In fact, differences in nuclear targeting in yeast as compared to plants will be interesting. Finally, in addition to carrying out these experiments in yeast because it is technically amenable and because it would allow us to compare our results to those obtained in plants, we also hoped to address the importance of nuclear localization of tRNA nucleotidyltransferase in yeast. Several experiments (Sarkar *et al.*, 1999; Hellmuth *et al.*, 1998; Feng and Hopper, 2002) have suggested the importance of nuclear localization of tRNA nucleotidyltransferase in yeast (perhaps in tRNA maturation and export). As there is no obvious nuclear localization signal in the yeast enzyme we hoped to address this question using the Arabidopsis tRNA nucleotidyltransferase if changing the apparent NLS did in fact alter localization.

4.3 Arabidopsis tRNA nucleotidyltransferase functions in yeast

Before being able to carry out any of the experiments that we proposed, it became crucial to prove that the *Arabidopsis* enzyme could complement a defect in the *CCA1* gene and adequately replace the tRNA nucleotidyltransferase in *S. cerevisiae*. We were confident that this would be the case as we had shown that a cDNA expressing the lupin tRNA nucleotidyltransferase could complement a temperature-sensitive defect in yeast (Shanmugam *et al.*, 1996) and that Arabidopsis tRNA nucleotidyltransferase-Gal4p fusion proteins could complement this same temperature-sensitive defect (Gu, 2000). Indeed, for the wild-type Arabidopsis CCA-adding enzyme, this was the case (Figure 6, panel A). Interestingly, as with the lupin enzyme (Shanmugam *et al.*, 1996), while the Arabidopsis protein was able to complement the nucleocytosolic defect it was not able to complement the mitochondrial defect as indicated by an inability of the cells to grow on the non-fermentable carbon source, glycerol (data not shown). This indicates that even when the amino terminal targeting signal, responsible for targeting this protein to mitochondria and plastids in Arabidopsis (Schmidt von Braun *et al.*, accepted), is contained in the protein it does not target to mitochondria in yeast. This is interesting as earlier studies on other proteins have shown that mitochondrial targeting signals from plants can function in yeast (Bowler *et al.*, 1989; Huang *et al.*, 1995; Smith *et al.*, 1994). In fact, this process appears to be more of a general phenomenon within different kingdoms since animal proteins also have been shown to be capable of being imported into yeast mitochondria and *vice versa* (*e.g.*, Lister and Whelan, 2006, Rawls *et al.*, 2000). This may suggest that there is something special about the targeting of a protein

that is shared between mitochondria and plastids in plants. Perhaps there are additional proteins involved in targeting tRNA nucleotidyltransferase in plants to ensure that it is delivered to both mitochondria and plastids. In this scenario we postulate that these accessory proteins are not found in yeast and so the protein does not get targeted to yeast mitochondria.

When plasmid shuffling was carried out to see if the Arabidopsis tRNA nucleotidyltransferase could function in the absence of any yeast tRNA nucleotidyltransferase, similar results were obtained: the enzyme was able to complement the nucleocytosolic defect (Figure 5 panel A) but not the mitochondrial defect (Figure 7). This indicates that the yeast tRNA nucleotidyltransferase is not necessary to interact with the Arabidopsis enzyme to help it exert its effect on the cell. The Arabidopsis enzyme is able to fold into an appropriate active conformation in the cell and function in the cytosol or perhaps the nucleus and the cytosol without any requirement of the native yeast enzyme for folding or localization. This also eliminates the possibility that the Arabidopsis enzyme itself is not active but was able to stabilize the ts form of the yeast enzyme sufficiently to allow it to confer viability to the ts strain.

When wild-type Arabidopsis tRNA nucleotidyltransferase-GFP fusion proteins were expressed in yeast, similar results were obtained (Figure 5 panel B). This indicates that attaching GFP to the carboxy-terminus of the Arabidopsis tRNA nucleotidyltransferase does not cause any conformational change or provide any steric impediment such that the Arabidopsis enzyme is unable to function. The conclusion to be drawn from these experiments is that the *Arabidopsis* tRNA nucleotidyltransferase can act as a substitute for the yeast enzyme in the nucleocytosolic compartment of the cell but

not in the mitochondrion so that it is reasonable to make observations about the biological role and importance of the NLS-like sequence in the Arabidopsis tRNA nucleotidyltransferase using the yeast model system (at least in the nucleocytosolic compartment).

In contrast, more interesting results were obtained when the same experiments were carried out using the Arabidopsis tRNA nucleotidyltransferase in which mutations had been introduced into the potential NLS. In this case none of these constructs were able to complement the mutant yeast tRNA nucleotidyltransferase (Figures 5 and 6). This indicated that these amino acids are important for the functioning of the Arabidopsis tRNA nucleotidyltransferase in yeast. However, whether the inability of these proteins to allow growth of yeast is due to misfolding impaired catalytic activity, or mislocalization remain to be shown.

4.4 Enzyme Activity Assay

As a first step to address how the changes in the potential NLS in the Arabidopsis protein alter the ability of this enzyme to complement a defect in the yeast *CCA1* gene, we tested whether these changes altered the activity of the enzyme. To do this we took advantage of a yeast strain with a temperature-sensitive mutation in its *CCA1* gene (Aebi *et al.*, 1990). While this strain grows at the permissive temperature (20°C), it cannot grow at the non-permissive temperature (37°C). This inability to grow at the non-permissive temperature results from a dramatic decrease in enzyme activity that is brought about by a single amino acid change in the enzyme (Shan *et al.*, submitted). This loss of enzyme

activity is evident in crude extracts generated from the mutant strain even at the permissive temperature shown in Figure 8. Aebi *et al.* (1990) showed only 4% CTP accepting activity and 3% ATP accepting activity in crude extracts isolated from the mutant strain as compared to an isogenic wild-type strain at 23°C. More recently, in our lab a specific activity at the permissive temperature of only about 5% for the mutant as compared to the wild-type enzyme was shown in an assay using purified enzyme (Shan *et al.*, submitted). In addition, we showed that the phenotype and loss of enzyme activity generated by the original Glu to Lys substitution could be replicated by a Glu to Phe substitution at the same position in the enzyme. Because of the reduced potential for reversion in the Glu (GAA) to Phe (TTT) substitution as compared to the Glu (GAA) to Lys (AAA) substitution the studies described here used the Glu to Phe variant enzyme for assays.

To carry out the enzyme assay on crude extracts, the incorporation of radioactive CTP was chosen as an indication of enzyme activity. CTP was selected over ATP as ATP is more likely to be a substrate for many other enzymes in the crude extract. Samples with and without added tRNA were used for each assay so that the incorporation of radiolabeled CTP into endogenous tRNAs by tRNA nucleotidyltransferase or into molecules other than tRNAs by additional enzymes in the crude extract could be determined. The basal enzyme activity, when no tRNA was added, showed modest levels of CTP incorporation (Appendix A) supporting our decision to study the incorporation of this nucleotide. As expected both the long (from ATG1) and short (from ATG3) isozymes of the Arabidopsis tRNA nucleotidyltransferase show activity, however, the variant enzymes had no activity above background (Figure 8). This result is interesting in

that it shows that the change of the KDTKGKSIPVVNHIFKFSMKRK sequence in the Arabidopsis protein to EDTEGESIPVVNHIFKFSMKEME results in a complete loss of enzyme activity. This is a surprising result as this sequence is found in a region of the protein not found in the functional *E. coli* enzyme (Figure 2) and which, based on available crystal structures for tRNA nucleotidyltransferases (Okabe *et al.*, 2003; Tomita *et al.*, 2006; Xiong *et al.*, 2003), appears not to interact with any other region of the protein or the substrates (Figure 12).

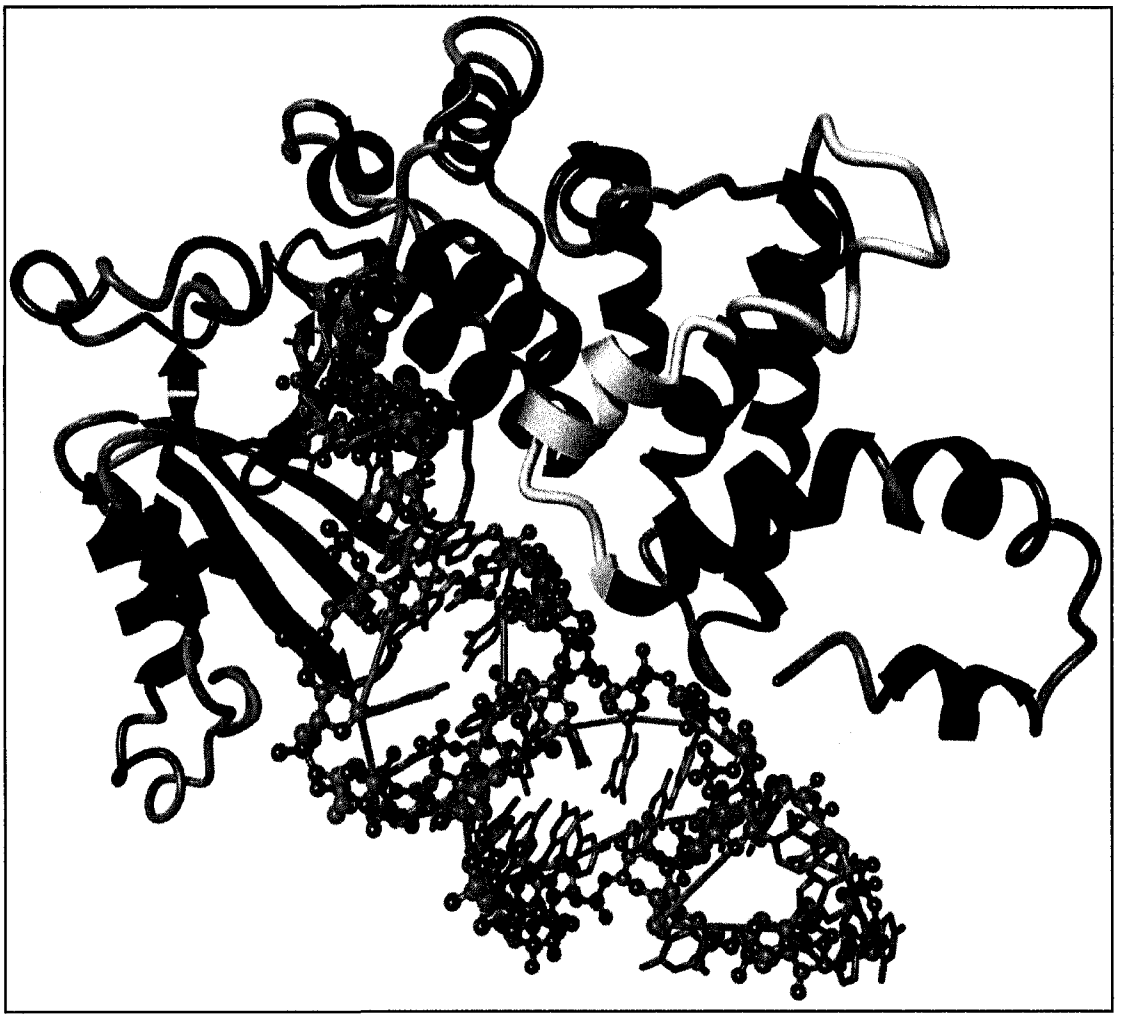


Figure 12: The homology model of the Arabidopsis tRNA nucleotidyltransferase (AT1G22660.1) based on the structure of a tRNA nucleotidyltransferase from *Aquifex aeolicus* (PDB:1VFG). The model shown starts at amino acid 105 and therefore does not contain the N-terminal targeting signal. The C-terminal ~150 amino acids could not be

modeled safely by homology, because of a very low sequence identity to the template within this region (<<10%). Glu 399 is the first amino acid of the altered motif and the altered region is shown in yellow. The tRNA and ATP analogue were added to the template to better illustrate the RNA-binding site (Schmidt von Braun *et al.*, accepted). Moreover, although we have changed the charge in this region (from overall positive to overall negative) we have maintained the hydrophilic nature of this region of the protein which helps to ensure that it remains on the surface and does not cause a major conformational change that will alter the protein's structure (Figure 12). So although all of the predictions suggested that this region of the protein would not be required for the production of a functional enzyme it is clear that it is. The question that remains is why this region of the protein is necessary for enzyme activity.

One possibility is that these amino acid changes alter the structure of the protein sufficiently that it is unable to fold correctly or is destabilized and degraded. Given the predicted location of these amino acids on the exterior of the protein and the type of substitutions that we made, this is unlikely. If the modified protein has undergone a conformational change such that its stability is compromised and it is rapidly degraded this might be seen by comparing the level of fluorescence in cells carrying tRNA nucleotidyltransferase-GFP fusion proteins. We showed (Figures 5 and 6) that fusing GFP to native tRNA nucleotidyltransferase did not inactivate it, suggesting that the tRNA nucleotidyltransferase contained in the fusion protein has folded into a conformation that maintains its activity and is stable. Therefore, the levels of the variant proteins could be compared to those of the wild-type proteins as measured by fluorescence in cells containing the fusion proteins. When this is done (Figure 9, compare E to G and F to H) it is clear that the variant enzymes actually show an increased fluorescence as compared to the wild-type. This suggests that the loss of activity does not result from a decrease in the

level of the tRNA nucleotidyltransferase although we cannot exclude the possibility that the increased GFP fluorescence seen in the cells carrying the variant proteins results from the unfolding or loss of the tRNA nucleotidyltransferase portion of the fusion protein such that GFP fluorescence is quenched to a lesser degree.

A second possibility is that these mutations do not destabilize the protein completely, but do alter the structure of the enzyme sufficiently that it becomes inactive. To address this question, detailed biochemical and biophysical (*e.g.*, enzyme kinetics, circular dichroism and fluorescence spectroscopy) would be required which would call for a purified protein and much additional work.

A third possibility is that despite our predictions, we have altered an amino acid directly responsible for substrate binding or catalysis. To test this hypothesis in appropriate detail would require purified tRNA nucleotidyltransferase and involved kinetic analyses and will be the subject of future experiments.

We began to address the second and third possibilities by more precisely defining the effects of the mutations that were introduced. In the original variants we altered two clusters of basic amino acids (indicated in bold here) separated from each other by thirteen amino acids (**K**DT**K**G**K**SIPVVNHIFKFSM**KRK**). Perhaps altering either of these clusters of basic amino acids alone would not alter enzyme activity. If one looks at the potential crystal structure (Figure 12) one might anticipate that the carboxy terminal basic amino acids in this sequence (...**KRK**) may be near enough to the tRNA substrate that converting them to EME could exert an effect on substrate binding. Similarly, perhaps the amino terminal basic cluster (**K**DT**K**G**K**...) is responsible for directing the nucleotide substrates (ATP and CTP) to the active site. The removal of either of these

clusters of positive charges and the introduction of negative charges instead may exert electrostatic repulsion on the negative charges contained in the tRNA or nucleotide triphosphates and reduce their ability to bind thus reducing enzyme activity. Likewise, if making both sets of these mutations is sufficient to alter the structure of the enzyme sufficiently to inactivate it, perhaps changing only one set would not exert such a dramatic effect. We found that altering either cluster of basic amino acids alone was sufficient to alter the enzyme sufficiently that it could not complement the temperature-sensitive defect in *S. cerevisiae* (Figure 10). So, each of these blocks of basic amino acids alone is necessary for enzyme structure or function.

Finally, even if this motif is required for enzyme activity, this does not exclude it from also functioning as a targeting signal. Hence studying the GFP fusion proteins may reveal any effect these changes have on localization. Therefore, we continued with an analysis of the localization of the GFP fusion proteins.

4.5 Localization of the tRNA nucleotidyltransferase in *S. cerevisiae*

The localization experiments for wild-type and variant tRNA nucleotidyltransferases in *S. cerevisiae* utilized a tRNA nucleotidyltransferase-GFP fusion protein. As discussed above, experiments with the wild-type protein showed that the fusion protein is able to complement a defect in the yeast tRNA nucleotidyltransferase indicating that the tRNA nucleotidyltransferase is still functional in this fusion protein and thus probably maintains its proper higher order structure. As a control for the localization of the fusion proteins in yeast we also looked at the

localization of GFP itself. The GFP control shows an equal distribution of fluorescence throughout the nucleus and cytosol of the cell (Figure 9 B I). This is in good agreement with what has been seen in the literature indicating that, when expressed in yeast, GFP is found both in the nucleus and the cytosol (Niedenthal *et al.,* 1996). This may be due to the small size of GFP (27 kDa) which allows it to diffuse into the nucleus across the nuclear envelope (Prasher *et al.,* 1992; von Arnim *et al.,* 1998) or due to the presence of cryptic nuclear localization information on the protein. Earlier experiments in Arabidopsis (von Arnim *et al.*, 1998) suggest that there is no cryptic NLS in GFP and that it enters the nucleus by diffusion. Therefore, if the nuclear localization we observe is due to simple diffusion then fusing GFP to tRNA nucleotidyltransferase (~27 KDa + ~66 KDa = >90 KDa) will eliminate that variable as this will surpass the diffusion limit for the nuclear pore complex (Feldherr and Akin, 1990). However, we cannot completely exclude the possibility that nuclear localization is due to a cryptic NLS in the GFP that we are using, so this complicates the interpretation of these data.

When the long or short forms of the Arabidopsis tRNA nucleotidyltransferase are fused to GFP and expressed in yeast we see fluorescence present in the nucleus as well as in the cytosol with slightly greater fluorescence in the nucleus than in the cytosol (Figure 9 E I and F I). The increase in fluorescence in the nucleus with the fusion proteins as compared to GFP alone (compare Figure 9, panel B I with panels E I and F I) indicates increased localization of the fusion protein to the nucleus which suggests that the Arabidopsis tRNA nucleotidyltransferase carries nuclear localization or nuclear retention information not found on GFP alone. Thus, we could conclude that the elevated nuclear

fluorescence levels are due to targeting information present within the tRNA nucleotidyltranferase and not due to targeting information contained on GFP alone.

When the NLS-modified long and short forms of the protein were fused to GFP and expressed in yeast, the most striking feature was that there appeared to be a definite augmentation in the amount of fluorescent signal present within the cells as a whole (Figure 9 G I and H I). Furthermore, the distribution of the fluorescence within the cells appears strikingly similar to that seen with the wild-type proteins, *i.e.*, the signal is found in both the cytosol and the nucleus but appears more intense in the nucleus. It seems that the relative amounts of GFP signal in the nucleus and cytosol has not changed between cells expressing the wild-type or variant proteins, but that the relative amount of protein increases in the variants. These results suggest that the sequence that we have classified as an NLS is not one, or is not the only NLS in this protein, as altering that sequence did not disrupt the localization of the tRNA nucleotidyltransferase-GFP fusion protein to the nucleus.

Another possible interpretation of the data observed for the modified proteins is that, in fact, there is more of the modified tRNA nucleotidyltransferase-GFP fusion protein in the nucleus as compared to the native protein (compare Figure 9, panels E I with G I and F I with H I, respectively). While such a result would challenge our original hypothesis that this sequence represents an NLS, since modification of the NLS should in theory inhibit the cell's ability to transport tRNA nucleotidyltransferase into the nucleus, several hypotheses may be put forward in order to explain such observations. Perhaps what we have called a nuclear localization signal is in fact a Nuclear Export Signal (NES). However, this is not likely given the fact that there is a fundamental difference in

the structure of NESs as compared to NLSs. NESs are usually leucine-rich sequences of amino acids (Elfgang *et al.*, 1999; Henderson and Eleftheriou, 2000; Kanwal *et al.*, 2002) that are recognized by non-bound receptors that facilitate the transport of the proteins containing these specific sequences. In some cases, although the consensus nuclear export sequence can be slightly altered by substituting the first leucine in the sequence with an isoleucine or valine (Kanwal *et al.*, 2002), it appears unlikely that the sequences that we suspected of being nuclear localization signals are, in fact, nuclear export signals since the main amino acid residues that we altered were charged, and our modifications maintained a charge on these sequences. In addition, if we are to assume that our sequence is an NES, then altering it should interfere with the transport of proteins from the nucleus to the cytosol, and thus we should observe a marked increase in the modified protein in the nucleus at the expense of the protein concentration in the cytosol. However, we do not observe this protein localization pattern since there is a significant increase in protein concentration both within the nuclear membrane and outside of it (compare Figure 9, panels E I with G I and F I with H I, respectively). The fact that there seems to be a uniform increase in these levels throughout the cell with no discernible shift in the pattern of isozyme distribution lends further support to the fact that the modified region of the protein plays a role other than as a targeting signal (Levis and Bourne, 1992).

One further explanation for the altered fluorescence observed may lie in the fact that the modified region of the tRNA nucleotidyltransferase may represent a degradation signal that facilitates the breakdown of this protein. Altering this region and perhaps removing a degradation signal could result in the increase in fluorescence as the concentration of the fusion protein increases. However, upon closer inspection, this

hypothesis also appears difficult to substantiate since the sequence that we mutated does not bear any close resemblance to well-known structures of degradation signals in proteins (Hill *et al.*, 1993). For example, the wild-type and variant enzymes do not differ at their N-termini, so the N-end rule (Davydov and Varshavsky, 2000) does not apply. Furthermore, our sequence also does not appear to be related to or is itself, what is known as a PEST sequence (Rechsteiner and Rogers, 1996) believed to be associated with proteins that have short intracellular half-lives due to the fact that PEST sequences appear to act as signals for the cell's degradation machinery, thus resulting in protein degradation (Rechsteiner and Rogers, 1996). The sequence that we changed does not initially appear rich in the amino acid residues that form PEST sequences (proline, glutamic acid, serine and threonine) and thus, it would be difficult to reconcile the notion that altering this sequence reduced the cell's ability to degrade the enzyme since this specific sequence was altered. In fact, it is the altered sequence that has a higher concentration of the amino acid residues (specifically glutamic acid) which form PEST sequences. However, the mutations that we did make result in the removal of five lysine residues from the protein. As ubiquitin is added to the ε-amino groups of lysine, perhaps we have altered the ubiquitin degradation pathway, such that the protein is less efficiently delivered to the proteosome (Hill *et al.*, 1993). This would have, of course, resulted in an increased protein concentration. However, it is not very clear whether the alteration of five to seven lysine residues out of the thirty-four lysines present within tRNA nucleotidyltransferase would be enough to elicit a significant effect on the ability of the ubiquitin pathway to efficiently turnover the mutant enzyme. Nevertheless, it is possible that a decrease of 14.7% in the number of lysines present may result in a decrease in the

number of ubiquitin binding sites present since this amino residue makes up an integral part of such sites. This will, in turn, have an effect on the amount of ubiquitin bound to tRNA nucleotidyltransferase, and thus the rate at which the cell's degradation machinery is able to turn over the protein.

Thus, perhaps the changes to the tRNA nucleotidyltransferase may have led to an augmentation in the observed fluorescence by removing a degradation signal from the protein, or also by resulting in a fusion protein that is more viable and biologically stable. It is difficult to reconcile this apparent increase in protein (as indicated by the increase in fluorescence) with the decrease in enzyme activity seen with the variant enzymes as compared to the wild-type. A possible explanation for this seemingly contradictory situation would be a structural change in the variant enzyme which, while boosting protein levels, interferes with the proper catalytic activity of tRNA nucleotidyltransferase. This would lead to a decrease in overall enzyme activity, however, the actual stability of the enzyme itself may remain unaltered or even increase due to the mutation and result in the augmented GFP fluorescence observed when the variant fusion protein is present. However, it is important to note that while it is the fluorescence of GFP that is measured, this does not provide an accurate insight as to what the final conformation of the fusion protein actually is. So, as discussed above, it is possible that the GFP fluorescence appears more intense not because there is more of the modified fusion protein in the cell, but because a conformational change in the altered fusion protein allows the GFP that it contains to fluoresce more intensely. Furthermore, the GFP itself may alter the character of the native or variant enzymes, so that what we see with the fusion proteins is not directly applicable to what was seen with the enzymes themselves

There is clearly a difference in the amount and distribution of the fluorescence signal that is not a function of tRNA nucleotidyltransferase, but of the changes in the amino acids in tRNA nucleotidyltransferase. Taken together all of these data suggest that the sequence that we modified is important to tRNA nucleotidyltransferase activity but is not a nuclear localization signal on this protein. Fluorescence data suggest that this sequence is not necessary for nuclear localization and in fact altering it results in an apparent increase in nuclear localization (compare Figure 9, panels E I with G I and F I with H I, respectively).

4.6 Comparative Localization Results

The studies described here were carried out in parallel with localization studies of Arabidopsis tRNA nucleotidyltransferase *in planta*. For example, a localization study in tobacco protoplasts showed a marked difference in tRNA nucleotidyltransferase-GFP localization depending on the amino-terminal targeting sequence present in the two *Arabidopsis* tRNA nucleotidyltransferase isoforms (Sabetti, 2002). In the first case where the shorter isoform originating from ATG3 and lacking the amino-terminal targeting sequence was transformed into the protoplasts there was an almost equal distribution of fluorescence throughout the cytosol and nucleus. In contrast, when the GFP fusion protein with the longer isozyme generated from ATG1 and containing the amino-terminal targeting signal was expressed in tobacco protoplasts, there was marked localization of fluorescence to discrete locales within the cytosol; indicating the presence of the enzyme within membrane-bound organelles such as mitochondria or plastids (Sabetti, 2002). In

yeast, the wild-type Arabidopsis tRNA nucleotidyltransferase lacking the amino terminal targeting information was found in both the cytosol and the nucleus (Figure 7) which is in good agreement with what was seen in tobacco protoplasts (Sabetti, 2002). In contrast, there was no mitochondrial localization in yeast.

Similar localization studies were done with Arabidopsis tRNA nucleotidyltransferase-GFP fusion proteins in onion cells (Schmidt von Braun *et al.*, accepted). The results of this study also agree well with my observations although there are some differences. When the long form of the Arabidopsis tRNA nucleotidyltransferase was fused to GFP and expressed in onion cells, the protein was found in mitochondria and plastids as expected since this protein contains the amino terminal targeting information. When the short form of tRNA nucleotidyltransferase was fused to GFP and expressed in onion cells it was distributed between the nucleus and cytosol as we observed in yeast. The most interesting observations come when the NLS-like sequence was altered. For the long form of the protein fused to GFP, this alteration did not increase the localization of the fusion protein to the cytosol or nucleus but did shift its distribution to plastids almost entirely. On the other hand, altering the NLS-like sequence for the short isoform fused to GFP in onion cells did not cause any significant difference in the localization of the isozyme to the nucleus except to increase its presence around the plasma membrane. These observations suggest that while the NLS-like sequence is important for mitochondrial targeting, it may not be integral for nuclear import.

4.7 Overview and future work

We set out to determine whether the candidate NLS-like sequence was, in fact, a true NLS or not. In order for the response to this investigation to be affirmative, it would have been necessary to demonstrate that this sequence was necessary and sufficient for nuclear localization. The results of both enzyme activity and localization studies on this NLS-like sequence indicate that it does not function as a nuclear localization signal in yeast and may have some alternate role in tRNA nucleotidyltransferase structure or function in Arabidopsis. While we originally set out to study the possible phenotype brought about by making mutations in this potential NLS, we observed a phenotype that cannot be attributed to defective targeting alone. While the distribution of the fluorescence signal appeared to change, there was still protein in both the cytosol and the nucleus such that it could have played its role in tRNA maturation in both locations. The fact that the mutation we utilized resulted in inactivated enzyme further shows that variables other than faulty localization may be responsible for the observed phenotype.

The results of the various assays and analyses performed as part of this project have implications with respect to subsequent studies in the field. First, the complementation studies prove the feasibility of studying modified *Arabidopsis* tRNA nucleotidyltransferase in the heterologous *S. cerevisiae* model system, a fact that greatly facilitates and simplifies such future studies for the various reasons mentioned above. Next, the fact that this study showed conclusively that the candidate sequence is not a true NLS allows future studies to focus their energies on other NLS-like regions. In fact, a direct consequence of this study was a subsequent experiment to modify a potential

NLS (KAKRQR) located at the carboxy terminus of the Arabidopsis tRNA nucleotidyltransferase (see Figure 2).

Future work will be required to define nuclear localization signals contained on the Arabidopsis tRNA nucleotidyltransferase and to define the role of the nuclear localization of tRNA nucleotidyltransferase in yeast.

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APPENDIX A

Experiment 1:

Plasmid	Background without tRNA	Average	With tRNA	Average	Difference	Absolute value of Difference	Relative activity (%)
pSCCA1	1206	992.5	18737	16221	15228.5	15228.5	100
	779		13705				
pKRYS-L	780	837.5	13558	12299.5	11462	11462	75.26677
	895		11041				
pKRYS-S	1115	763.5	11235	11920.5	11157	11157	73.26395
	412		12606				
pKRYS-ΔL	1007	722	1267	766	44	44	0.288932
	437		265				
pKRYS-ΔS	807	778	714	763	-15	15	0.0985
	749		812				
Vector	119	181	111	481.5	300.5	300.5	1.973274
G131-2	243		852				

Experiment 2:

Plasmid	Background without tRNA	Average	With tRNA	Average	Difference	Absolute Value of Difference	Relative activity (%)
pSCCA1	4228	3497	19574	22007	18510	18510	100
	2766		24440				
pKRYS-L	486	391	18427	18141	17750	17750	95.89411
	296		17855				
pKRYS-S	545	943	17460	18300	17357	17357	93.77093
	1341		19140				
pKRYS-ΔL	1077	897.5	630	487.5	-410	410	-2.21502
	718		345				
pKRYS-ΔS	386	885	189	176.5	-708.5	708.5	-3.82766
	1384		164				
Vector G131-2	1718	1028	539	417.5	-610.5	610.5	-3.29822
	338		296				

Overall results:

Plasmid	**relative activity overall average of two experiments (%)**
pSCCA1	100
pKRYS-L	85.58044
pKRYS-S	83.51744
pKRYS-ΔL	-0.96304
pKRYS-ΔS	-1.96308
Vector G131-2	-0.66247